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(54) **Novel hybrid regulatory region.**

(57) A hybrid regulatory region capable of directing and regulating transcription of a gene sequence positioned downstream therefrom which comprises the promoter sequence of a first promoter-operator region fused to an operator sequence of a second promoter-operator region from which the promoter sequence has been removed wherein said operator sequence can regulate the promoter from said first region more efficiently than can its native operator sequence. In a specific embodiment the hybrid regulatory region comprises the phage λ promoter P_R fused to the operator region of λ promoter P_L.

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EP 0 138 437 A3

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EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	EP-A-0 067 540 (GENENTECH INC.) * Whole document *	1	C 12 N 15/00 C 12 N 1/20 // C 12 N 1/10 C 12 R 1/19
A	MOLECULAR AND GENERAL GENETICS, vol. 163, 1978, pages 197-203, Springer-Verlag, DE; J. HEDGPETH et al.: "Lambda phage promoter used to enhance expression of a plasmid-cloned gene" * Page 198: "Plasmid construction" *	1-3	

TECHNICAL FIELDS SEARCHED (Int. Cl. 4)

C 12 N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	06-11-1986	CUPIDO M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
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O : non-written disclosure
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T : theory or principle underlying the invention
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NOVEL HYBRID REGULATORY REGION

Technical Field

This invention relates to the construction of a regulatory sequence which provides a new operator region to regulate transcription initiation while leaving the promoter intact. This regulatory sequence can be used for the regulated transcription and translation of prokaryotic or eukaryotic genes.

Background of the Invention

The expression of a gene in both prokaryotic and eukaryotic organisms involves first the synthesis of RNA from a DNA template followed by protein synthesis from the RNA.

Transcription, the synthesis of RNA from a DNA template and the first step in the expression of a gene, is controlled by certain signals present on the DNA. These signals are nucleotide sequences which initiate transcription and control the amount of transcription taking place at a given time. The control signals generally consist of promoter and operator regions. The promoter region is a site that is specific for the binding of RNA polymerase and is the initiation point for transcription. Operators function in conjunction with a repressor to control the amount of transcription.

Transcription of a DNA segment is effected by the enzyme RNA polymerase. After RNA polymerase binds to the promoter at the -35 and -10 recognition regions (M. Rosenberg and D. Court, Ann. Rev. Genet. 13:319-353, 1979), it transcribes nucleotides which encode a ribosome binding

site and translation initiation signal and then transcribes the nucleotides which encode the actual structural gene until it reaches so-called stop signals at the end of the structural gene. The RNA polymerase acts by moving along the DNA segment and synthesizing single-stranded messenger RNA (mRNA) complementary to the DNA. As the mRNA is produced, it is bound by ribosomes at the ribosome binding site (also called the Shine-Dalgarno region). The ribosomes translate the mRNA, beginning at the translation initiation signal and ending at the stop signals, to produce a polypeptide having the amino acid sequence encoded by the DNA.

Through the use of genetic engineering techniques genes from one organism can be removed from that organism and spliced into the genetic information of a second organism and the polypeptide encoded by that gene expressed by the second organism. It is desirous to maximize the expression of the foreign gene and thus obtain high yields of the resultant polypeptide. It has been realized that one way in which gene expression can be regulated is through selection and manipulation of the control signals discussed above.

There is variation among different promoters in their strength and their ability to be repressed efficiently. A promoter which cannot be repressed easily is of only limited use with genes whose protein product in small amounts is toxic to the cell or inhibits maintenance of the plasmid. In such situations, maximal repression of the genes is needed to assure that the host cell and/or plasmid can grow normally until derepression is desired.

Some promoters also suffer a disadvantage when they are present on multi-copy plasmids in that they cannot be repressed efficiently unless a suitable repressor also is located on that plasmid and thus present in multiple copies.

Such promoters are in contrast to others which can be repressed fully by the amount of repressor made from a single chromosomal gene copy. These promoters, however, may have other drawbacks. They may not, for example, be as strong as other promoters.

Various efforts have been made to manipulate different promoter/operator systems so as to enhance promoter strength or increase efficiency of repression. European Patent Application 067,540 (see also De Boer et al. in "Promoters: Structure and Function," ed. R.L. Rodriguez, M.J. Chamberlin, Praeger, 1982, pp. 462-481), for example, describes and claims a hybrid promoter/operator. This hybrid is constructed by ligating the -10 region of one promoter/operator sequence, capable of being derepressed by induction, downstream from a DNA fragment which comprises the -35 region and 5' flanking region of a second promoter which has a stronger signal sequence than the first promoter/operator sequence. The two DNA fragments are linked at a position between about the -35 and -10 recognition sequences for binding of RNA polymerase to the promoter/operator sequence. The fusion results in an entirely new promoter sequence.

Although such a hybrid promoter/operator can be used advantageously in certain situations, it still may prove to be unsatisfactory in others. For example, although the transcription efficiency of the promoter contributing the -10 region may be enhanced, the promoter may not be regulated as tightly as desired under certain circumstances.

There thus remains a need for a regulatory sequence that has a strong promoter which can be repressed highly efficiently. Accordingly, it is an object of this invention to construct a novel regulatory region having these characteristics. It also is an object of this invention to construct such a regulatory region that can be

ligated conveniently to a variety of prokaryotic and eukaryotic genes.

According to the present invention we provide a hybrid regulatory region capable of directing and regulating transcription of a gene sequence positioned downstream therefrom which comprises the promoter sequence of a first promoter-operator region fused to an operator sequence of a second promoter-operator region from which the promoter sequence has been removed wherein said operator sequence can regulate the promoter from said first region more efficiently than can its native operator sequence.

Description of the Drawing

Figure 1 depicts the promoter/operator sequences which are fused together to make the hybrid O_L/P_R regulatory region.

Figure 2 depicts a map of a plasmid containing the hybrid O_L/P_R regulatory region.

Figures 3, 4 and 5 illustrate the steps in the construction of plasmid pGX2606, which contains the O_L/P_R region.

Figures 6, 7 and 8 illustrate the steps in the construction of plasmid pGX1043.

Detailed Disclosure of the Invention

The present invention relates to a hybrid promoter/operator region capable of directing and regulating transcription of a gene sequence positioned downstream therefrom which provides an intact strong promoter ligated to a new operator region which can regulate transcription initiation more efficiently than the promoter's own natural operator(s). The hybrid regulatory region of this invention is constructed from two promoter/operator regions, a first region which contains a strong promoter and a second region which contains an efficient operator. These regions are cleaved and fragments taken from them are fused together such that the resultant hybrid region comprises the complete promoter

sequence of the first region and the efficient operator of the second.

To make this novel hybrid region the first region generally is cleaved at a restriction enzyme recognition site located upstream from the complete promoter sequence and the second region is cleaved at a site downstream from its operator sequence. The appropriate fragments from each of these two regions then are fused together in accordance with conventional methods so as to form the novel hybrid regulatory region of this invention. Alternatively, the first region may be cleaved at a restriction enzyme recognition site that is within the nucleotide sequence of the promoter provided that when the resulting fragment containing the partial sequence of the promoter is fused to the operator sequence of the second region, the nucleotide sequence at the 3' end of the operator region is such that the complete nucleotide sequence for the promoter will be restored.

The two regions may be cut at a naturally occurring common or complementary restriction enzyme recognition site or at a common or complementary site which has been introduced into one or both of the regions by in vitro mutagenesis. Alternatively, if the DNA fragments taken from the two regions have noncomplementary ends, a synthetic DNA segment which matches the restriction sites of the fragments can be prepared and used to link the two fragments.

The details of this invention will be set forth below in terms of a particular embodiment of this invention. It is to be understood, however, that this is done for illustrative purposes only and is not to be construed as limiting.

In one embodiment of this invention the hybrid regulatory region is constructed from two phage λ promoter/operator regions. These two promoters of phage λ,

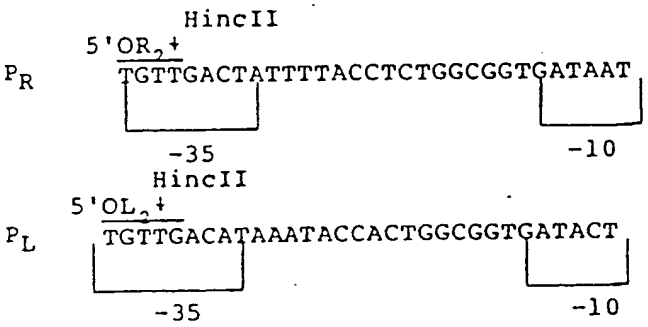
which function early in λ infection, are known as P_R and P_L (Eisen, H. and M. Ptashne, The Bacteriophage Lambda, A.D. Hershey, ed., Cold Spring Harbor Lab, N.Y., 1971, pp. 239-270). The P_R sequence provides a strong promoter, but the promoter cannot be repressed as efficiently (i.e., to as low a level) as λ promoter P_L (Queen, C.J., Mol. Appl. Genet. 2: 1-10 (1983)). A second disadvantage of the P_R promoter is that when it is present on multi-copy plasmids it can be repressed efficiently only when a λ repressor also is located on the plasmid and thus in multiple copies. When, however, the λ repressor is also present on the plasmid, complete derepression of λP_R cannot be achieved efficiently unless the temperature is raised to 42°C. In contrast, the P_L promoter can be repressed fully by the amount of repressor made from a single chromosomal gene copy, and derepression is effective at 37-38°C. The lower induction temperature is useful for proteins which may be rendered less active by heating at 42°C.

The structure of two segments of the λ genome containing promoters P_R and P_L is diagrammed in Figure 1. RNA polymerase binds to each promoter at the -35 and -10 regions (Rosenberg, M. et al., Ann. Rev. Genet. 31: 319-353 (1979); Hawley, D.K. et al., Nucl. Acids Res. 11: 2237-2255 (1983)). The ability of RNA polymerase to bind each promoter is antagonized by the λ repressor (cI protein) which binds at operator sites O_L 1, 2 and 3 and O_R 1, 2 and 3 (Ptashne, M. et al., Cell 19:1-11 (1980)).

As shown in Figure 1, the P_L and P_R regions have a naturally occurring common HincII site. The regions are cut with endonuclease HincII, then a fragment from each region is fused together, such that the sequence upstream from the HincII site (to the left of HincII in Figure I) is the P_L fragment and the sequence downstream from the HincII site (to the right of HincII in Figure I) is the

P_R fragment. The hybrid region has been designated O_L/P_R.

The HincII site in P_L and P_R is located within the -35 region of each promoter. When the P_L and P_R segments are fused at the HincII site, the new regulatory region recreates the exact and complete sequence of P_R, for the bases upstream of the HincII cut site are identical in P_L and P_R (Rosenberg et al., supra; Hawley et al; supra).



Similarly, the fusion at the HincII site recreates O_L2, a portion of which is shown above, because the G residue in O_L2 to the right of HincII is also found in O_R2. The O_L/P_R hybrid has the repressor binding characteristics of P_L. The primary repressor binding sites O_R1 and O_L1 have identical DNA sequences (Pirrotta, V. Nature 254:114 (1975); Humayun, et al., J. Molec. Biol. 112: 267 (1977)); thus, the differences between P_R and P_L in their ability to be repressed apparently resides in the differences between the remaining repressor sites. The O_L/P_R hybrid made in accordance with the above-discussed procedure contains the O_L2 and O_L3 repressor sites and the repressor binding characteristics of P_L. The O_L/P_R hybrid thus can be repressed to the low basal levels of O_L. Furthermore, the O_L/P_R regulatory region can be repressed efficiently when the λ repressor gene (cI) is located on the chromosome of the bacterial

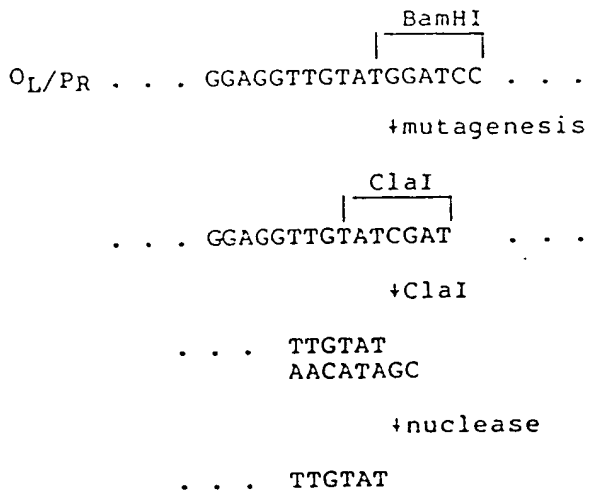
host and derepressed efficiently at temperatures less than 42°C.

In a specific embodiment of the invention, the P_L fragment is derived from the plasmid pGW7 (provided by Geoffrey Wilson) which contains a segment of the λ genome. The P_R segment is derived from plasmid pCQV2 (Queen, C., J. Mol. Appl. Genet. 2:1-10, 1983). pCQV2 contains an alteration in a segment of the λ DNA sequence such that a BamHI site overlaps the ATG of the cro gene, the first gene downstream from P_R. When the BamHI site is cleaved and the resulting single stranded region removed, an ATG codon is present at the blunt end of the hybrid promoter/operator region. The resulting O_L/P_R hybrid regulator has been cloned into a plasmid designated pGX2606 (see Figure 2). An E.coli cell culture transformed with this plasmid has been designated GX3123 and deposited with the Northern Regional Research Laboratory, Peoria, Illinois, as NRRL No. B-15551.

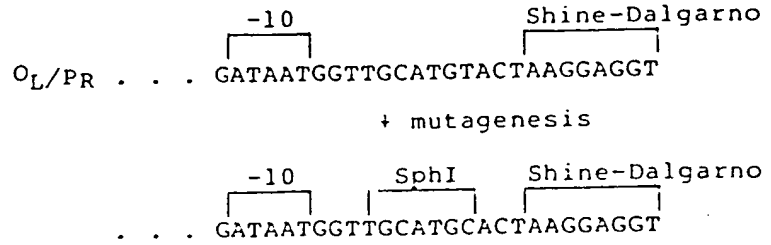
In this example, the promoter can be repressed by maintaining the plasmid in an E.coli cell which carries the gene for wild type λ repressor on the chromosome. Alternatively, if the plasmid carrying the O_L/P_R region is introduced into a cell which has the gene specifying the temperature-sensitive λ repressor mutant, cI857, repression is maintained at 30°C. Induction of the cI857 lysogen is obtained by raising the temperature to 37-42°C to allow expression at a desired time (Campbell, A., The Bacteriophage Lambda, ed. A.D. Hershey, Cold Spring Harbor Lab, N.Y., 1971, pp. 13-44). Nonregulated expression of the gene of interest linked to O_L/P_R also can be obtained by putting the plasmid into a non-lysogen. With this variation, gene expression is constitutive, and the temperature can be maintained at 37°C which is the optimal growth temperature for E.coli.

The hybrid regulatory region of this invention provides a translation initiation region derived from the region between the promoter and the first gene downstream from the promoter in the plasmid from which it was derived, which can be joined to a gene sequence to provide all needed translation initiation signals for E.coli. This includes the ribosome binding site, known as the Shine-Dalgarno region (Shine, J. and L. Dalgarno, Proc. Natl. Acad. Sci. USA, 71:1342-46, 1974) and the ATG. As discussed above, for example, the end of the O_L/P_R region proximal to the P_R promoter can be digested so as to provide a blunt end with an ATG (translation initiation codon) at the terminus. The region then can be fused to a gene lacking an ATG.

Alternatively, the region proximal to the 3' end promoter in this hybrid can be altered such that the promoter region no longer carries an ATG codon for translation initiation and so can be fused to genes which carry their own initiation codon. An example of this using the O_L/P_R hybrid is shown by converting the BamHI site to a ClaI site by site directed mutagenesis in vitro (Zoller, M.J., et al. in Methods in Enzymology (in press))



In a third embodiment of this invention, a single base change made with <u>in vitro</u> mutagenesis can be used to create a restriction site downstream from the -10 RNA polymerase recognition site of the hybrid regulatory region. Such a cut separates the hybrid promoter/operator from the Shine/Dalgarno region (Shine, J. and L. Dalgarno, <u>supra</u>, preceding the first downstream gene, thus allowing the insertion of any other natural or synthetic Shine/Dalgarno sequence. These substitutions provide additional possibilities for high expression. One example shows the insertion of an <u>SphI</u> site in the O_L/P_R at such a position by site directed mutagenesis (see above).



The hybrid promoter/operator regulatory region can be used for transcription and translation of various

prokaryotic or eukaryotic genes either in a regulated or an unregulated form. The efficient repression which can be obtained with such a hybrid makes it especially useful for fusion to genes whose protein products are toxic to the cell in small amounts or inhibit plasmid maintenance. Maximal repression of the expression of such genes enables the cells to grow normally and to retain the plasmid until derepression is desired. Expression of the genes then can be induced when cell viability no longer is important.

The following examples are intended to further illustrate this invention and are not to be construed as limiting.

I. Cloning of λP_L and λP_R Fragments Into Intermediate Vectors

A. Cloning of P_L from PGW7 into pUC8 (Fig. 3)

Plasmid of pGW7 (8007 base pairs, obtained from Geoffrey Wilson) contains a 3987 base pair segment of bacteriophage λ DNA from nucleotides 34498 to 39173 (excluding bases 38104 to 38754 which were deleted). The numbering of the residues in λ DNA is from Sanger, F. et al., J. Mol. Biol., 162, 729-773 (1982). This region contains the early λ promoter P_L from which was isolated a fragment from endonuclease sites BglII to HindII (HincII) (bases 35615 to 35711).

Plasmid pGW7 DNA (10µg) was digested with 11.2 units endonuclease BglII (New England Biolabs, Inc.) for 3 hours at 37°C in "medium salt" restriction buffer (50mM NaCl, 10mM Tris, pH 7.4, 10mM $MgSO_4$, 1mM dithiothreital). The 5566 base pair fragment was isolated after electrophoresis in a gel of 1% low melting agarose (Bethesda Research Laboratores, Inc.) in E buffer (50 mM Tris, pH 7.5, 30mM sodium acetate, 3mM EDTA) and extracted from the agarose with butanol as described by Langridge et al., Anal.

Biochem. 103, 264-271 (1980). The DNA was precipitated by addition of 2.5 volumes ethanol and pelleted in an SW40 Beckman ultracentrifuge rotor at 4°C and 35,000 rpm for 1 hr. The pellet was dried in vacuo and suspended in 200µl H_2O.

The isolated 5566 base pair fragment (10µλ) was digested with 8 units endonuclease HindII (Boehringer Mannheim, Gmbh) in medium salt buffer for 20.5 hrs. at 37°C. The digest was extracted with phenol and ether and subjected to electrophoresis on a 6% polyacrylamide gel (acrylamide:bisacrylamide - 30:1) in TBE buffer (90 mM Tris, pH 8.3, 90 mM boric acid, 4 mM EDTA). After staining the gel with ethidium bromide, the desired 110 base pair fragment was cut out and removed from the gel by electroelution in 400 µl 0.1X TBE. One ml 0.2M NaCl, 20 mM Tris, pH 7.4, 1mM EDTA was added and the DNA was purified by passage over an Elutip (Schleicher and Schnell, Inc., Keene, N.H.) as suggested by the manufacturer. The DNA was precipitated with ethanol as above and pelleted in a Beckman SW28 ultracentrifuge rotor at 25000 rpm for 1 hr at 4°C. The pellet was dried in vacuo and suspended in 20µl H_2O.

Plasmid pUC8 (Vieira J. and J. Messing. Gene, 19 259-268, 1982), 10 µg, was digested with 9.1 units endonuclease Hind II (Boehringer Mannheim, GmbH) for 60 min. at 37°C, then another 9.1 units of enzyme was added and incubated another 15 hrs. at 37°C. The DNA was precipitated in 0.3M sodium acetate, pH 5.5, with 2.5 volume ethanol. The dried pellet was suspended in 16 ml H_2O, to which was added medium salt buffer and 20 units endonuclease BamHI in a total reaction volume of 20 µl. The reaction was incubated for 2 hours at 35°C and then extracted with phenol, precipitated with ethanol, and resuspended in 10 µl H_2O.

For ligation of the P_L fragment to pUC8, approximately 5 ng fragment was joined to approximately 30

ng pUC8 in a 20 µl reaction containing 200 units T4 DNA ligase (New England Biolabs, Inc.), 10 µg/ml bovine serum albumin (Bethesda Research Laboratories, Inc.) 0.5mM ATP, 50mM Tris, pH 7.8, 10mM $MgCl_2$, 20 mM dithiothreital. The reaction was carried out for 23 hours at 12°C.

E.coli K12 JM103: F' traD36 $proA^+B^+$ $lacI^q$ lacZΔM15/Δ(lac pro) supE thi rpsL4 sbcB15 cndA) was grown in YT broth (5g yeast extract, 8g trypstone, 5g NaCl per liter H_2O) and made competent for transformation by $CaCl_2$ treatment (Cohen, S.N. et al., Proc. Natl. Acad. Sci USA, 69, 2110-2114, 1972). Two 200µl samples of competent cells (approx. $2x10^9$/ml) were each added to 8µl ligation mix and kept on ice 40 min. The mix was heat shocked at 42°C 2 min., diluted 15-fold in YT broth, incubated at 37°C 1 hr., and plated on selective medium (YT broth with 1.5% Difco agar, 2µg/ml ampicillin, 2ml/l 0.1 M isopropylthio-β-D-galactoside [IPTG], 2ml/l 5-bromo-4-chloro-3-indolyl-β-D galactoside [Xgal]. Ligations which produced plasmids containing the insert were indicated by a color change in the colony in the medium. This method for detecting inserts is described in more detail by Vieira, J. and J. Messing Gene 19, 259-269, 1982.

After 15/hrs incubation at 37°C, 85 colonies were obtained. Miniprep DNA was prepared from white colonies by the method of D.S. Holmes and M. Quigley Anal. Biochem. 114:193-197 (1981).

To verify that a 96 bp fragment had been inserted into pUC8, miniprep DNA was digested with two endonucleases whose sites border the insert on each side. 0.5µg DNA in a total volume of 20 µl was incubated with 8 units endonuclease HindIII (Boehringer Mannheim GmbH) in medium salt buffer for 1 hr. at 37°C, then for another 4 hrs at 37°C with an additional 8 units HindIII. The reaction was stopped by heating for 5 minutes at 65°C. It was brought to 50mMTris, pH 7.4, 100 mM NaCl in a volume of

35 µl and digested further with 20 units endonuclease EcoRI (New England Biolabs, Inc.) for 15 minutes at 37°C. A 5µl sample was analyzed by electrophoresis on a 5% polyacrylamide gel in TBE buffer. By digesting with EcoRI and HindIII a 118 base pair fragment should be obtained if the correct 96 base pair λP_L fragment has been inserted between them. The correct isolate was identified as having a fragment which comigrated with a 119 base pair marker. The identity of the insert was confirmed by DNA sequencing (Maxam, A. M. and W. Gilbert Methods in Enzymology, ed. L. Grossman, K. Moldave, Academic Press, N.Y. vol. 65, pp. 499-559 (1980)), from DNA which had been extracted from cells by a method similar to the detergent lysis procedure (Molecular Cloning, ed. T. Maniatis, E. F. Fritsch, J. Sambrook, Cold Spring Harbor Laboratory, N.Y. p. 92, 1982). The DNA was purified on two CsCl-ethidium bromide gradients by established procedures and passed over a column of Biogel A-50 (BioRad Laboratories).

B. Cloning of P_R from pCQV2 into pUC9 (Fig. 4)

These procedures were carried out in a manner analogous to the procedures described in section A; therefore, only specific changes will be noted here. All other details can be assumed to be the same as in section A.

Plasmid pCQV2 (Queen, C. J. Mol. Appl. Genet. 2, 1-10, 1983) contains λDNA from base numbers 37169 to 38043 and it was modified to contain an endonuclease BamHI site overlapping the ATG of the λ cro gene. From pCQV2 was isolated a HindIII to BamHI fragment which contains most of P_R and the Shine-Dalgarno region (Shine and Dalgarno, supra) preceding the λ cro gene.

pCQV2 (50µg) was digested with 50 units endonuclease BamHI (Bethesda Research Laboratories) in medium salt buffer at 37°C for 1 hr. Endonuclease HindII (Boehringer

Mannheim, GmbH) then was added (80 units) and digestion was continued 20.5 hrs. at 37°C. The digest was extracted with phenol and ether and subjected to electrophoresis on a preparative 6% polyacrylamide gel. The 50 base pair BamHI to HindII fragment was removed from the gel by electroelution, passed over a Schleicher and Schuell Elutip and precipitated with ethanol.

The vector pUC9 is similar to pUC8 except that the cloning sites from EcoRI to HindIII are in the opposite orientation (Vieira, J. and J. Messing Gene 19, 259-269, 1982) pUC9 (10µg) was digested with endonuclease BamHI and HindII as described before. Approximately 15 ng digested pUC9 was joined to 0.2 ng P_R fragment in a reaction with 200 units T4 DNA ligase for 23 hrs. at 12°C in a reaction volume of 20µl.

Competent E.coli K12 JM103 cells were transformed with 8µl of the ligation and plated on YT agar plates + IPIG + X-gal + ampicillin at 37°C. After 15 hrs. incubation, there were 326 white colonies. Miniprep DNA was prepared from some of these, and it was digested with EcoRI and HindIII sites on either side of the insert. The insert (50 base pairs) was removed in this way to give a 72 base pair diagnostic fragment. DNA from an isolate with the correct size insert was purified and sequenced by the Maxam-Gilbert technique to confirm its identity.

The cloning of the P_L and P_R fragments into pUC8 and pUC9 resulted in orienting the fragments in the same direction and in placing useful endonuclease sites on either side of the inserts. pUC8 containing P_L is hereafter referred to as pGX2602 and pUC9 containing P_R as pGX2603.

C. Joining of the P_L and P_R Fragments and Cloning of the Joined Piece (Fig. 5)

Purified DNA (25µg each) of pGX2602 and pGX2603 was digested with 24 units of endonuclease HincII (same as HindII, New England Biolabs, Inc.) in medium salt buffer 2 hrs at 37°C; another 24 units of enzyme were added and incubation continued at 37°C for 1 hour (pGX2602) or 4 hours (pGX2603). The digested DNAs were precipitated with ethanol and resuspended in medium salt buffer. pGX2602 was then incubated with 56 units endonuclease HindIII (New England Biolabs, Inc.) and pGX2603 was incubated with 25 units endonuclease PstI (Takara Inc., Japan) at 37°C for 2 hrs. The two DNA samples were then mixed, extracted with phenol, and precipitated with ethanol. The digestion of both DNAs with two different enzymes allowed fewer possible combinations when they were joined in the next step. The desired junction was of P_L to P_R at the HincII site.

For joining of the linearized plasmid, the DNA (50µg) was treated with 2000 units T4 polynucleotide ligase (New England Biolabs, Inc.) in a volume of 100µl for 15 hr. at 16°C. Another 2000 units of ligase was added and incubation was continued for another 48 hrs.

An EcoRI to BamHI fragment which was thought to contain the left operator fused to P_R was removed from the joined linear DNA fragments and cloned into another plasmid. This was done by first digesting the DNA with 100 units endonuclease EcoRI (New England Biolabs, Inc.) at 37°C for 2 hrs. and precipitating it with ethanol. The pellet was suspended in 96µl 100mM Tris, pH 8.0 and digested with 944 units (4µl) bacterial alkaline phosphatase for 40 min. at 65°C to remove 5' phosphate groups. After three extractions with phenol and an ethanol precipitation, the free ends were labeled with γ^{32}p-ATP by incubating in 50mM Tris, pH 7.4, 10mM $MgCl_2$, 5mM dithiothreital with 10 units T4 polynucleotide kinase (P.L. Biochemicals Inc.) and 100µCi ^{32}p-ATP (Amersham, Inc. 6300 Ci/m mol) at 37°C for 35 min. Unlabeled ATP was added to

1mM and incubated for 10 min at 37°C. The mixture was extracted with phenol, and the DNA was precipitated with ethanol. The DNA was then digested with 80 units endonuclease BamHI in medium salt buffer for 2 hrs. at 37°C, extracted with phenol and precipitated with ethanol. The pellet was suspended in 45µl TBE + dyes (80% glycerol, 0.5% bromphenol blue, 0.5% xylene cyanol) and loaded onto a 3mm thick 6% polyacrylamide preparatory gel. The gel was made from 11.2 ml acrylamide (40%; 30:1 acrylamide: bis-acrylamide), 56 ml H_2O, 7.5 ml 10X TBE, 0.5ml 10% ammonium persulfate and 55µl TEMED (BioRad Laboratories, Inc.). After electrophoresis at 250V for 1 hr., the gel was stained with ethidium bromide, and the 150 base pair EcoRI to BamHI fragment was excised, removed from the gel by electroelution, passed over a Schleicher and Schuell Elutip and precipitated with ethanol. The amount of material at this point was barely detectable by ethidium bromide staining, therefore, the fragment was hereafter detected on gels by autoadiography since it was end-labeled with ^{32}P.

The plasmid pGX1025 was used as the vector for cloning of the O_L/P_R fragment. It was digested with endonucleases EcoRI and BamHI under conditions described previously, and then it was treated with bacterial alkaline phosphatase to remove 5' phosphates and thereby to permit recircularization of the plasmid only when it was joined to the O_L/P_R fragment.

Conditions for ligation of the O_L/P_R fragment to the vector were as follows: 200 units T4 DNA ligase (New England Biolabs), 500 ng pGX1025 prepared as described above and the entire recovered O_L/P_R fragment (amount unknown) under standard reaction conditions and a 20µl total volume. Incubation was at 16°C for 18 hrs.

The host for transformation of the ligated DNA was E.coli K12 JM101(λ) F'traD36 proA$^+$B$^+$ lacI^q lacZΔM15/Δ(lac pro) supE thi. Cells (200 µl) were made

competent and transformed by 8µl ligation mixture as described for JM103(λ). The transformed cell suspension was divided into 200µl aliquots and plated on LB agar (1.0% tryptone, 0.5% yeast extract, 1.5% agar, all from Difco Laboratories, 0.5% NaCl) + 100 µg/ml ampicillin at 37°C for 15 hrs. Approximately 6000 transformed colonies were obtained.

Miniprep DNA was prepared (Holmes and Quigley, supra) from 64 colonies grown to saturation in 10ml LB (broth minus agar). The plasmid DNA was extracted twice with phenol, precipitated with ethanol, and suspended in 100µl 10mM Tris, 1mM EDTA, pH 8.0. A sample of each miniprep DNA, 5µl in a total volume of 20 µl, was digested with 12 units endonuclease HincII (New England Biolabs, Inc.) in medium salt buffer for 2 hrs at 37°C. Two isolates had a diagnostic piece of 50-60 base pairs when the digest was analyzed by electrophoresis on a 5% polyacrylamide minigel. This HincII fragment originated from the HincII site internal to the O_L/P_R fragment and from a HincII site just 3' to the insert in the vector plasmid. Another diagnostic test was to digest 5µl miniprep DNA with 16 units endonuclease BamHI (New England Biolabs, Inc.) in medium salt buffer for 2 hrs. at 37°C. The completion of the BamHI digestion was confirmed by electrophoresis of a small portion of the digest on a 1% agarose minigel. The digest was then brought to 100mM NaCl, 50 mM Tris, pH 7.4 and digested with 20 units endonuclease EcoRI for 2 hrs at 37°C. The mixture was analyzed by electrophoresis on a 5% polyacrylamide gel. The BamHI and EcoRI sites flank the O_L/P_R insert; therefore, this digestion should yield a fragment of 164bp. The two isolates which had the correct HincII fragment also had the correct BamHI to EcoRI fragment.

In order to confirm the identity of the O_L/P_R insert, DNA was purified from one isolate which had the

correct restriction pattern and subjected to DNA sequencing by the technique of Maxam and Gilbert. The sequence was identical to that of the corresponding segments from phage λ (Sanger, F., et al. supra).

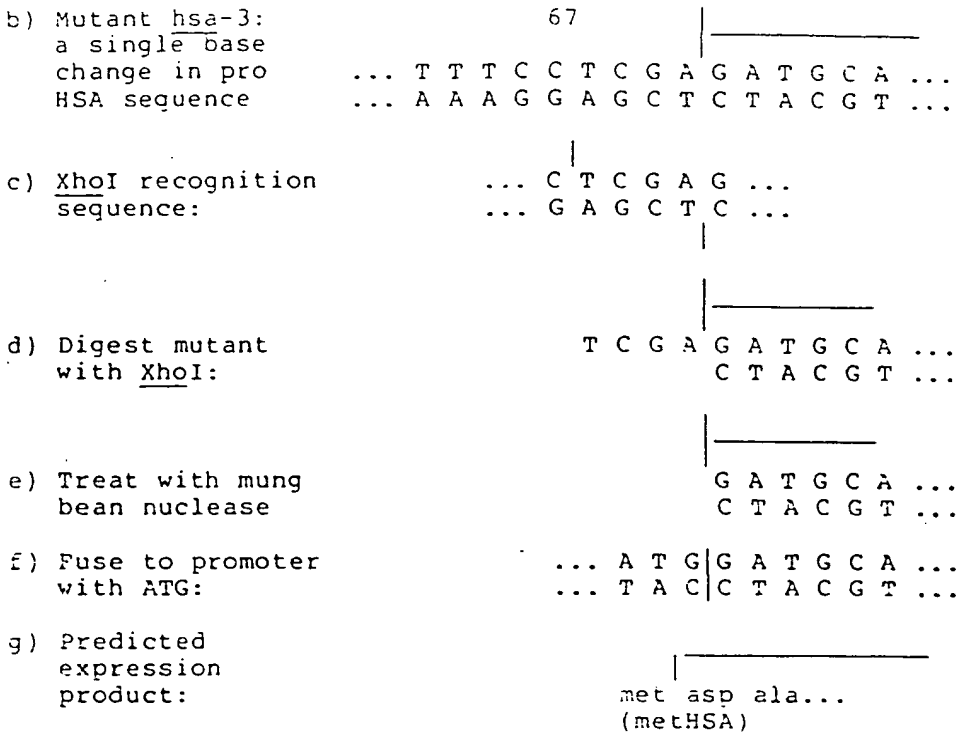
The plasmid containing O_L/P_R has been designated pGX2606. An E.coli culture transformed with this plasmid has been designated GX3123 and Deposited with the Northern Regional Laboratory as NRRL No. B-15551.

Example II

Expression of Human Serum Albumin Gene Under the Control of the OL/PR Regulatory Region Insertion of an XhoI Cleavage Site Preceding the Sequence Coding Mature Human Serum Albumin (HSA)

The O_L/P_R hybrid region was used to regulate expression of a human serum albumin (HSA) gene. In this procedure, the O_L/P_R regulatory region supplied the promoter, Shine-Dalgarno region, and ATG codon for translation initiation. The O_L/P_R region was ligated to a mature HSA coding sequence which contained no ATG codon at its 5' end. This form of HSA was created by introducing a restriction site (XhoI) which overlapped the codon for the first amino acid of HSA. Oligonucleotide-directed mutagenesis was used to modify the wild type sequence coding for preproHSA in order to place an XhoI restriction endonuclease cleavage site overlapping the 5' end of the mature HSA coding sequence. The strategy for this mutagensis and for expression of metHSA in E.coli from this modified sequence is outlined in the following diagram and described below.

		start mature HSA codons
a) Wild type	63	
hsa-1	... T T T C G T C G A G A T G C A ...	
DNA sequence	... A A A G C A G C T C T A C G T ...	



The mutagenesis was accomplished in the following steps, adapted from Zoller, M. and M. Smith (supra).

1. A portion of a human serum albumin gene was subcloned into the bacteriophage M13mp8, as shown in Fig. 6. Purified DNA from plasmid pGX401, containing a full length HSA clone with pre-pro sequences (designated hsa-1) was digested with HincII and the 1.35 kb fragment comprised of hsa-1 sequences from nucleotides -22 to 1328 was purified by electroelution from an agarose gel. M13mp8 was digested with HincII and treated exhaustively with bacterial alkaline phosphatase (BAP) to remove 5' phosphates. BAP-treated M13mp8 DNA was incubated with the purified hsa-1 HincII fragment in the presence of T4 DNA ligase at 12°C (1.35:1 molar ratio of vector to insert). The ligation mix was used to transfect E.coli strain JM103. The hsa-1 sequence could be inserted into M13mp8 in either clockwise or counterclockwise orientation such that the

single-stranded viral DNA from the recombinants would contain either the sense or nonsense strand of hsa-1. To determine the orientation of the insert, plaques were screened by hybridization with oligomer probes complementary to a portion of the sense or nonsense strands of hsa-1 (as described in detail below). An isolate in which the hsa-1 fragment had been inserted in the desired orientation was confirmed by restriction endonuclease mapping and by DNA sequencing from the 3' HincII site toward the XbaI site. The phage containing the cloned hsa-1 fragment was designated MGX-2.

2. The desired mutant differed from the wild type sequence by a single nucleotide. A 17 base oligonucleotide was synthesized which was complementary to the wild type sequence except for a single base mismatch at the position of the desired base change (G--> C).

3. The mutagenic oligonucleotide was used as a primer for DNA synthesis with DNA polymerase I. After treatment with DNA ligase the product heteroduplex closed circular DNA molecules were purified by alkaline sucrose gradient centrifugation, pooled, dialyzed, and used to transfect competent E.coli.

4. The plaques obtained were screened by hybridization of phage DNA to the mutagenic oligonucleotide. The principle behind this procedure is that the oligonucleotide used to direct the mutagenesis will form a duplex of higher thermal stability with mutant DNA, to which it is perfectly matched (17 of 17 base pairs), than it will to DNA of a wild type clone, to which it is imperfectly matched (16 of 17 base pairs). Therefore the mutant phage can be differentiated from wild type phage in a hybridization experiment under conditions which discriminate between perfectly matched oligomers and mismatched oligomers (R.B. Wallace, M.J. Johnson, T. Hirose, T. Miyake, E.H. Kawashima, and K. Itakura, Nucl.

Acids. Res. 9:879, 1981). Phage stocks were prepared from individual plagues. 20 µl of each phage supernatant was spotted onto nitrocellulose filter paper using an S & S Minifold™ device (96 well capacity) to concentrate the 20 µl onto a small area of the filter. Samples were applied in duplicate to make identical 4 x 12 arrays.

The filter was air dried and baked in vacuo at 80°C for 2 hours. This filter was prehybridized and then hybridized with 5' end labeled oligomer (10 pmol in 4 ml) as described in Zoller and Smith, supra. After one hour of hybridization at 25°C, the filter was removed from the probe solution and rinsed for 2 minutes in 50 ml 6XSSC at 25°C. The filter was cut horizontally to separate the identical arrays. The top half of the filter was washed at 48°C for 10 minutes (2X25 ml 6XSSC) and the bottom half at 52°C for 10 minutes (2X25 ml 6XSSC). Filters were air dried and exposed to X-ray film for 12 hours at room temperature. It was determined that hsa-1 DNA (MGX2) formed mismatched hybrids with the mutagenic oligonucleotide in 1 M salt at 25°C which were stable during washes at 48°C but unstable at 52°C. Therefore, duplicate DNA samples from plaques obtained after mutagenesis were hybridized at 25°C and then were washed at 48°C and 52°C.

5. Double-stranded replicative form DNA was prepared from two hybridization-positive (A7,D7) and two hybridization-negative (A8,D8) clones. Each DNA was tested for the presence of an XhoI cleavage site. DNA from phages A7 and D7 was cleaved by XhoI; DNA from phages A8 and D8 was not. The correct location of the XhoI site in the DNA from phages A7 and D7 was confirmed by digestion with various other restriction enzymes. DNA sequence analysis confirmed the desired base change had occurred. This variant of hsa is called hsa-3, and the M13 clone bearing it is called MGX4. MGX4 has a restriction site which will

cleave precisely at the 5' end of the mature HSA coding sequence.

Reconstruction of hsa-3 in a Plasmid Vector

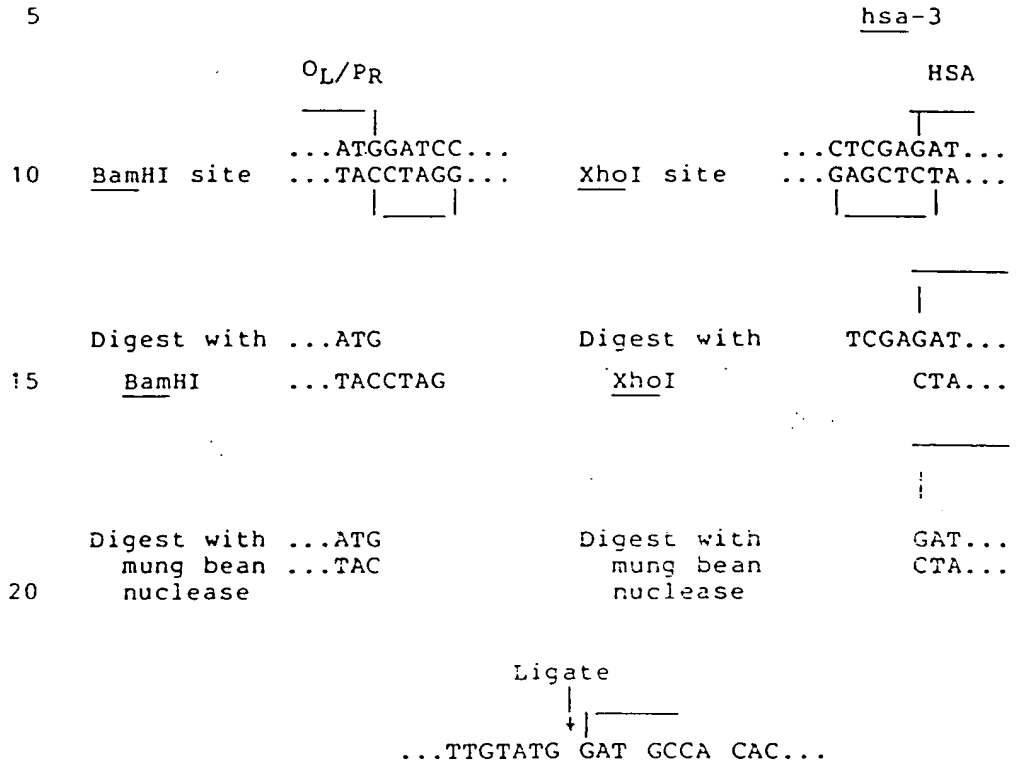
The hsa-3 gene was constructed in a plasmid vector suitable for the addition of expression signals. Plasmid pGX1031 contains all of the hsa-1 clone from pGX401, except a small section of the prepro region (3 codons). It was used to provide the 3' end of the gene and other necessary vector components. Figure 7 outlines the procedure used to fuse the 5' portion of the hsa-3 gene from MGX4 to the 3' end of the hsa-1 gene in pGX1031 in order to make pGX1042 containing hsa-3 with the XhoI site. pGX1031 (Figure 7) was cut with EcoRI and XbaI, and the fragment shown was purified. This fragment was mixed with vector MGX4 DNA cut with the same enzymes, and the mixture was incubated with DNA ligase. After transformation of E.coli JM101 with the ligation mixture, 1200 ampicillin resistant transformants were obtained. Plasmid DNA from 45 of these which were randomly chosen was characterized by digestion with several restriction endonucleases, including XhoI. The plasmid designated pGX1042 was determined to have the desired construction.

Construction of pGX1043 Containing the OL/PR Regulator Linked to hsa-3 at the XhoI Site

The outline for the fusion of O_L/P_R to hsa-3 is shown in Figure 8. The bacterial host for the transformation was JM101(λ). The O_L/P_R promoter should be repressed in this strain. As fragments for this construction were not purified, the steps described below were performed for reducing the number of parental molecules and one type of recombinant plasmid which otherwise would have been recovered. It thus was expected

that the desired transformant would be highly enriched among the colonies recovered.

The following outline illustrates how the junction between the promoter and hsa-3 was made.



Plasmid pGX2606 DNA was prepared by digestion with BamHI (Rice, R.H. and G.E. Means, J. Biol. Chem. 246:831-832 (1971)). The 5' single-stranded ends were removed by mung bean nuclease, and the plasmid was cut again with BglI. In order to prevent recircularization of pGX2606 in the subsequent ligation, the DNA was treated with bacterial alkaline phosphatase. Plasmid pGX1042 DNA was cut with XhoI, treated with mung bean nuclease to remove the 5' single-stranded ends, and cut with BglI.

Approximately 250 ng of each plasmid DNA was mixed and incubated with T4 DNA ligase at 16°C for 18 hours. The ligation mixture was cut with BamHI to linearize any

pGX1042 plasmid which had recircularized and to linearize one of the possible recombinant types.

Approximately 75 ng of ligated DNA was used to transform competent JM101 (λ). The transformation mixture was plated on medium containing ampicillin and incubated at 37°C. 430 transformants were obtained.

The final plasmid pGX1043 was expected to have the sequence listed (at the bottom of the figure above) at the junction between promoter and hsa-3. The sequence to the left of the arrow including the ATG and the Shine-Dalgarno region (underlined) came from the O_L/P_R segment. The sequence to the right of the arrow came from hsa-3.

The 430 transformants obtained were tested in several ways.

A. Colony hybridization (M. Grunstein and D.S. Hogness Proc. Natl. Acad. Sci U.S.A. 72:3961, 1975). A ^{32}P-labeled probe from the 5' end of hsa was used to detect colonies which carry hsa. The transformants were grown in LB medium plus 100 µg/ml ampicillin in 96 well microtiter plates at 37°C. Aliquots were transferred with a replicator to nitrocellulose filters on LB + ampicillin plates where they were incubated a further 5 hr at 37°C. The conditions for processing the filters and doing the hybridization are described in the above reference. The ^{32}P-labeled DNA probe was prepared from a plasmid containing the sequence for the 5' end of mature HSA. A 178 base pair fragment from the 5' end was labeled with γ-^{32}P-ATP using T4 polynucleotide kinase, and purifying the desired hsa fragment on a 5% polyacrylamide gel. Known positive and negative controls gave the expected results. 39% of the transformants had at least this segment of hsa.

B. Southern blot (E.M. Southern, J. Mol. Biol. 98: 503, 1975). Since the host cells were lysogenic for λ, the transformants could not be tested directly for the λ O_L/P_R sequence by colony hybridization. Instead, DNA

from 45 transformants which did have hsa sequences (identified in step A above) was prepared, plasmid DNA was separated from chromosomal DNA on an agarose gel, and a Southern blot was prepared from this gel. The correct plasmid DNAs were identified by hybridization to a ^{32}P-labeled O_L/P_R fragment, made by end labeling the 164 base pair EcoRI to BamHI fragment from pGX2606. Hybridization was carried out as in A. 44 isolates had the O_L/P_R sequences.

C. Identification of correctly-constructed plasmid. Plasmid DNAs from each of the 45 transformants tested in step B were analyzed by restriction endonuclease digestion. Two clones appeared to have the proper construction according to: 1) analysis of the size of the undigested plasmids by agarose gel electrophoresis, 2) lack of a BamHI site (the pGX1042 parent has a BamHI site but the desired recombinant does not) and 3) presence of restriction fragments diagnostic for the presence of the O_L/P_R regulator.

D. DNA sequencing. Two of the plasmid DNAs which had all the expected characteristics described above were subject to sequencing in phage M13. M13 subclones of the O_L/P_R-hsa-3 fusion from pGX1043 were constructed by cloning the O_L/P_R-hsa-3 segment (EcoRI to HindIII) from pGX1043 into M13mp9 (EcoRI to HindIII). Dideoxy DNA sequencing was performed by the method of Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977). An isolate which had the predicted sequence was termed pGX1043.

Expression of metHSA

In order to test for expression of HSA, plasmid pGX1043 was transferred to strain GX1864 which carries the temperature inducible, defective prophage λHlΔBam cI857. Transcription was then induced from the O_L/P_R

promoter by raising the temperature to 42°C, and samples taken at different times were analyzed. The samples were subjected to electrophoresis in SDS-polyacrylamide gels (U. Laemmli Nature 227:6880, 1970) followed by the Western blot procedure (H. Towbin et al. Proc. Natl. Acad. Sci. U.S. 76:4350, 1979, W. Burnette Anal. Biochem. 112:195, 1981.) HSA was assayed using anti-HSA antibody followed by goat anti-rabbit antibody coupled to horseradish peroxidase. A color development procedure was used to visualize the antigen bands. Controls of the host strain as well as uninduced cells containing pGX1043 showed no stainable bands. Induced pGX1043 DNA gave rise to a major band with a mobility corresponding to a molecular weight of 68 kilodaltons (kd). There were also minor bands with higher mobilities corresponding to lower molecular weights. These minor bands could arise from proteolytic degradation of HSA or from abnormal transcription or translation starts and stops in the hsa gene.

By comparing the intensity of the stained 68kd band from pGx1043 with known amounts of pure HSA (Sigma Chemical Co.), it was estimated that 0.2% of the total protein in extracts of induced pGX1043 was HSA after 2 hours induction. This amount of expression was confirmed by performing immunoprecipitation from extracts labeled with H-leucine during induction as before. Known amounts of HSA (fraction V Sigma Chemical Co.) labeled with ^{14}C-formaldehyde were used as an internal standard (Rice, R.H. and G.E. Means). The standard was added to cell extracts which were then immunoprecipitated by the method of S.W. Kessler (J. Immunol. 115:1617-1624, 1975) with minor modifications. The immunoprecipitate was subjected to electrophoresis on a 7.5% polyacrylamide gel and the HSA band was cut out and ozidized in a Packard sample oxidizer. The $^{14}C\ O_2$ and $^{3}H_2O$ products were separately quantitated by liquid scintillation spectrometry. The yield of ^{3}H-HSA was

determined by direct comparison to the yield of added known amounts of HSA-^{14}C standard. The amount of ^{3}H-HSA was then calculated as a percentage of the total ^{3}H leucine incorporated into bacterial protein. The maximum yield of HSA was 0.2% of the total protein.

An E.coli culture transformed with this plasmid has been designated GX1864 (pGX1043) and deposited with the Northern Regional Research Labortory, Peoria, Ilinois, as NRRL No. B-15613.

Claims

1. A hybrid regulatory region capable of directing and regulating transcription of a gene sequence positioned downstream therefrom which comprises the promoter sequence of a first promoter-operator region fused to an operator sequence of a second promoter-operator region from which the promoter sequence has been removed wherein said operator sequence can regulate the promoter from said first region more efficiently than can its native operator sequence.

2. The hybrid regulatory region of claim 1 which comprises the phage λ promoter sequence of P_R fused to the operator sequence of phage λ P_L promoter-operator region, from which the promoter sequence P_L has been removed.

3. The hybrid regulatory region of claim 1 which comprises a fragment from the phage λ P_R promoter-operator region containing the intact promoter P_R and the operator site O_R1 fused to a fragment taken from the phage λ P_L promoter-operator region containing operator sites O_L2 and O_L3.

4. The hybrid λ regulatory region of claims 2 or 3 wherein the terminus proximal to the P_R promoter has been altered so as to provide a blunt end with a methionine (ATG) translation initiation codon at the terminus such that the hybrid region can be fused to a gene which lacks a translation initiation codon.

5. The hybrid λ regulatory region of claims 2 or 3 wherein the terminus proximal to the P_R promoter has been altered so that it lacks an ATG codon at the terminus such that it can be fused to genes which carry their own initiation codon.

6. The hybrid λ regulatory region of claims 2 or 3 wherein the region has been digested with a restriction enzyme so as to remove the native Shine-Dalgarno region located downstream from the P_R promoter sequence of the region.

7. A plasmid comprising the hybrid regulatory region of claim 4 and capable of directing transcription and translation of said gene sequence in a prokaryotic or eukaryotic organism.

8. The plasmid of claim 7, wherein said prokaryotic organism is of the genus Escherichia.

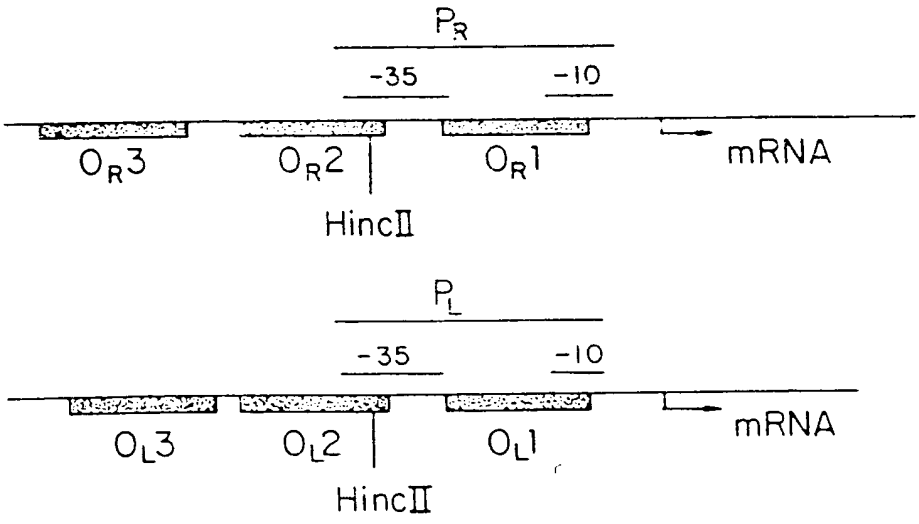
9. A microorganism transformed by the plasmid of claim 7.

10. The microorganism of claim 9 of the genus Escherichia.

11. The microorganism of claim 10 of the species coli.

12. A microorganism of the genus and species E.coli, designed as GX 3123 and deposited with the Northern Regional Research Laboratory as NRRL No. B-15551.

FIG. 1


P_R
-35
-10
O_R3
O_R2
O_R1
mRNA
HincII
P_L
-35
-10
O_L3
O_L2
O_L1
mRNA
HincII

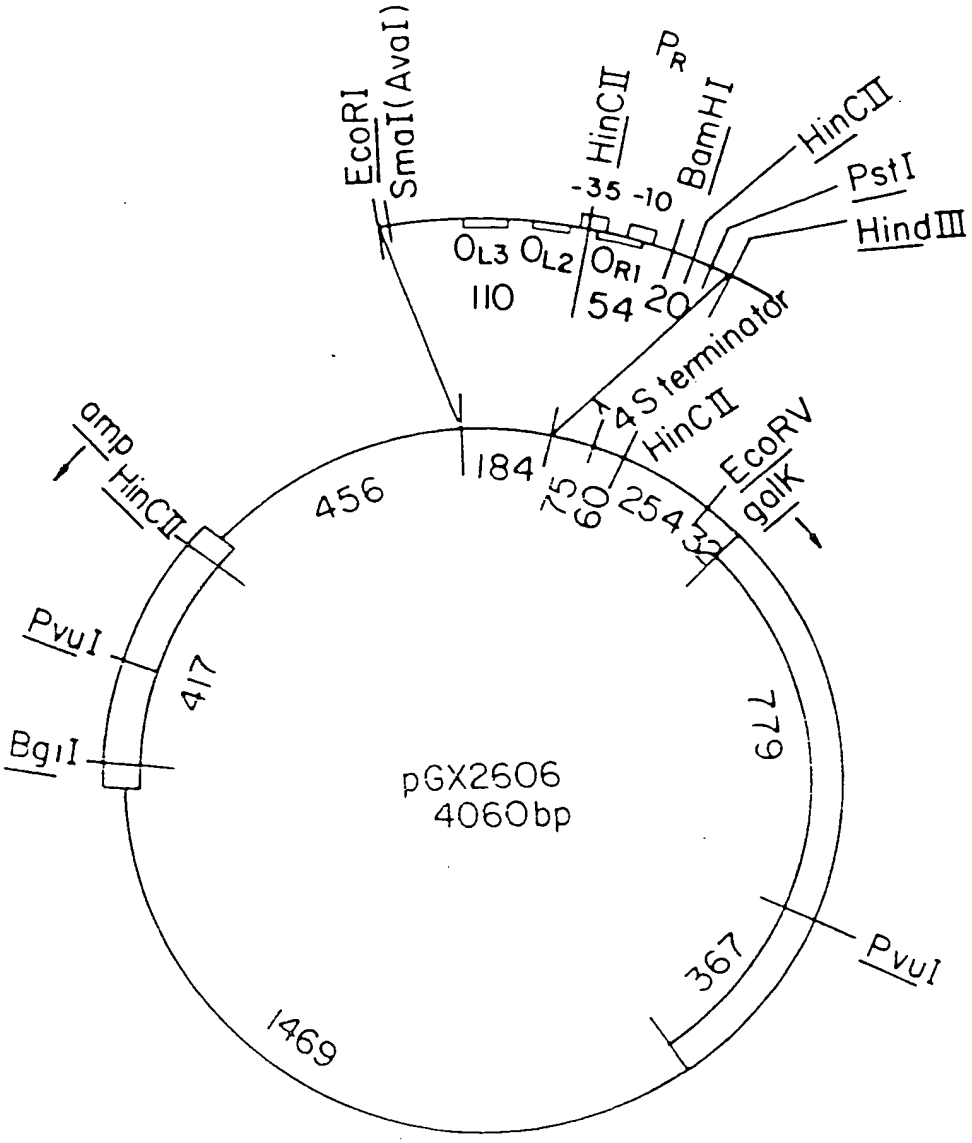

EcoRI
SmaI(AvaI)
HinCII
-35 -10
P_R
BamHI
HinCII
PstI
HindIII
O_{L3} O_{L2} O_{R1}
110
54 20
λ terminator
amp
HinCII
184
4S terminator
HinCII
EcoRV
galK
456
75
60
254
35
PvuI
417
779
Bgl I
pGX2606
4060bp
367
PvuI
1469

FIG. 2

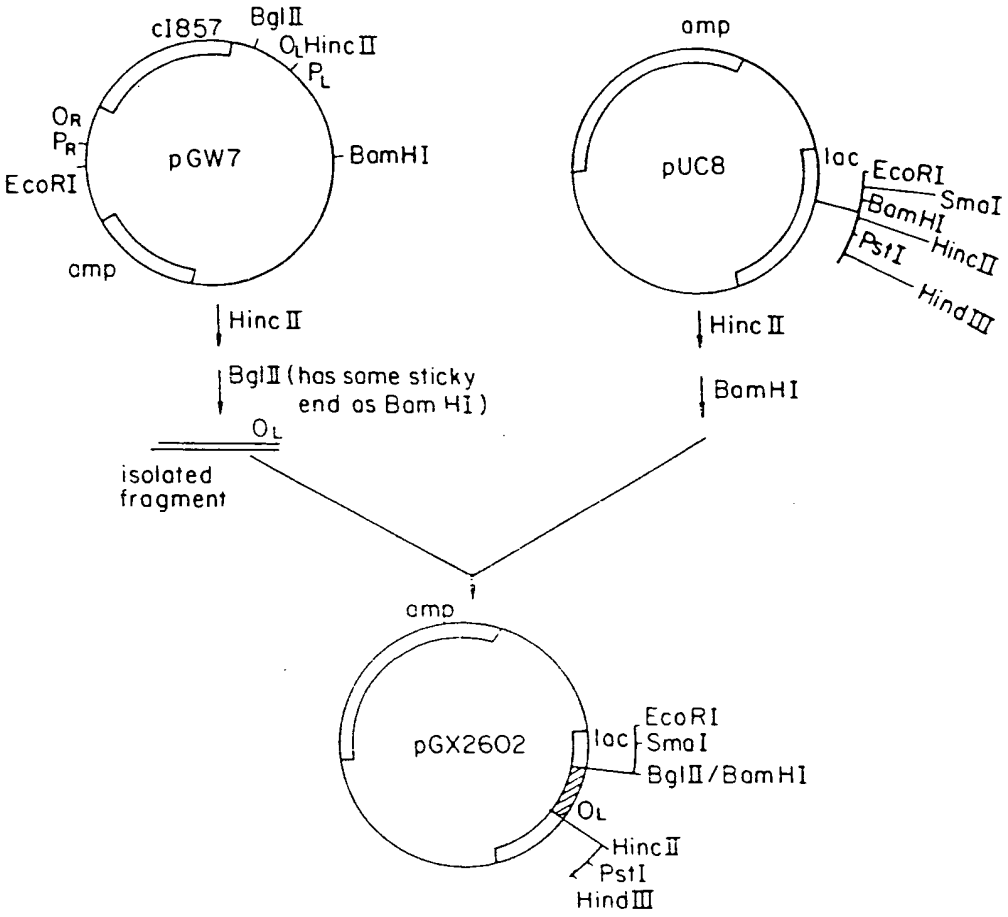
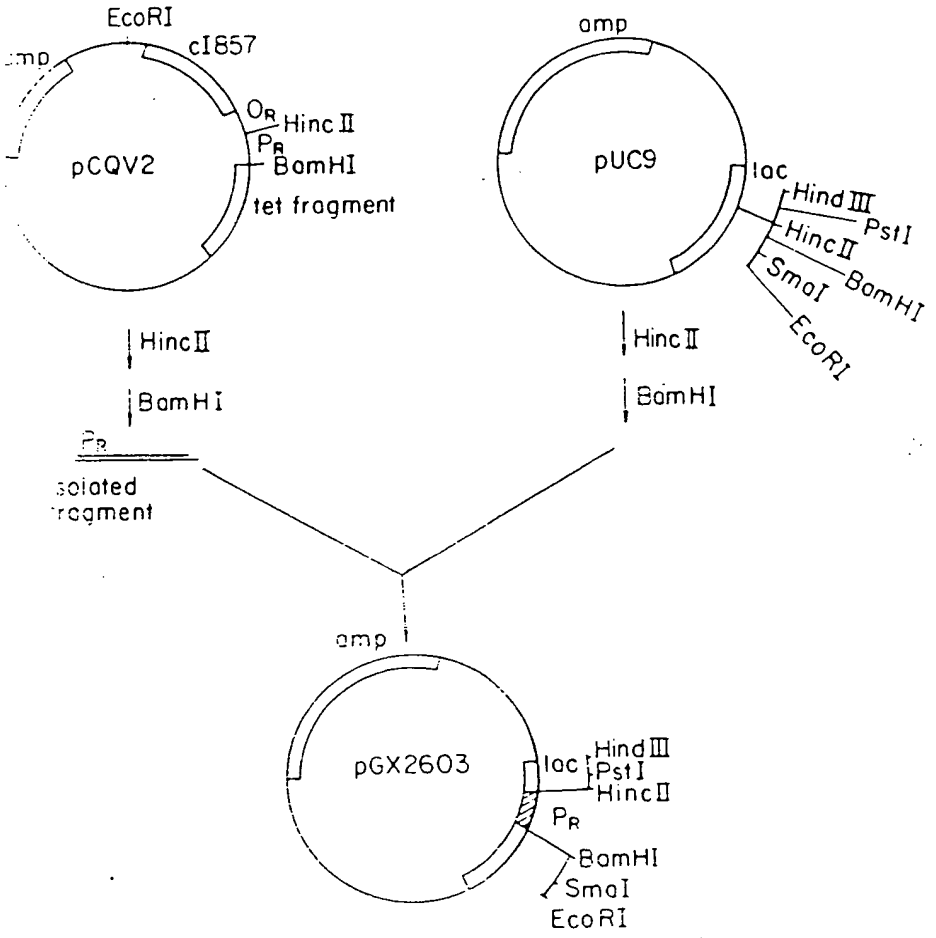

cI857
BglII
O_LHincII
P_L
O_R
P_R
EcoRI
pGW7
BamHI
amp
HincII
BglII (has some sticky end as BamHI)
O_L
isolated fragment
amp
pUC8
lac
EcoRI
SmaI
BamHI
HincII
PstI
HindIII
HincII
BamHI
amp
pGX2602
lac
EcoRI
SmaI
BglII/BamHI
O_L
HincII
PstI
HindIII

FIG. 3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cI857
amp
O_R
Hinc II
P_R
BamHI
pCQV2
tet fragment
Hinc II
BamHI
P_R
amp
pUC9
lac
Hind III
Pst I
Hinc II
Sma I
BamHI
Eco RI
Hinc II
BamHI
amp
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lac
Hind III
Pst I
Hinc II
P_R
BamHI
Sma I
Eco RI

FIG. 4

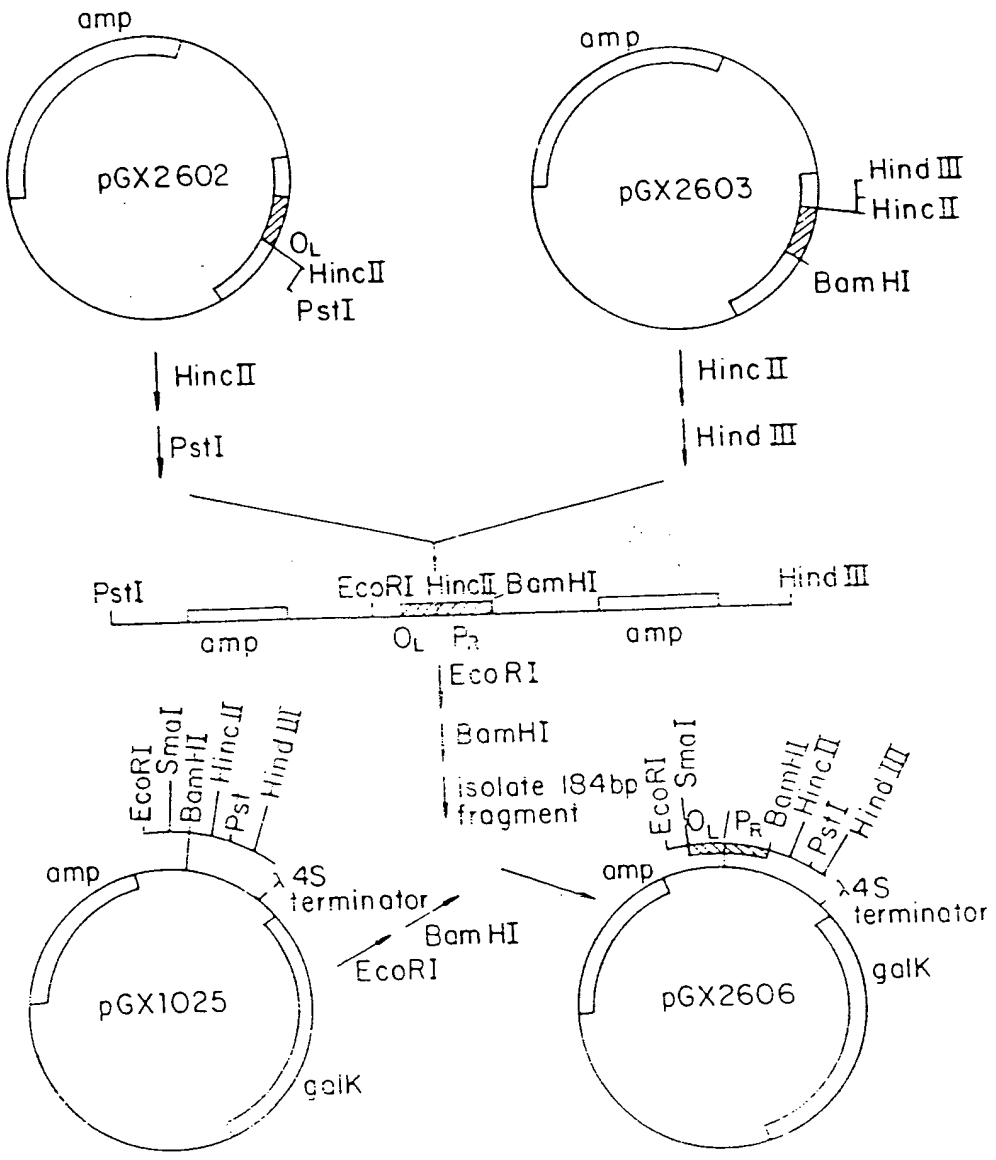

amp
pGX2602
O_L
HincII
PstI
amp
pGX2603
Hind III
HincII
BamHI
HincII
PstI
HincII
Hind III
PstI
EcoRI HincII BamHI
Hind III
amp
O_L P_R
amp
EcoRI
BamHI
isolate 184bp fragment
EcoRI
SmaI
BamHI
HincII
PstI
Hind III
amp
λ 4S terminator
BamHI
EcoRI
pGX1025
galK
EcoRI
SmaI
O_L/P_R
BamHI
HincII
PstI
Hind III
amp
λ4S terminator
galK
pGX2606

FIG. 5

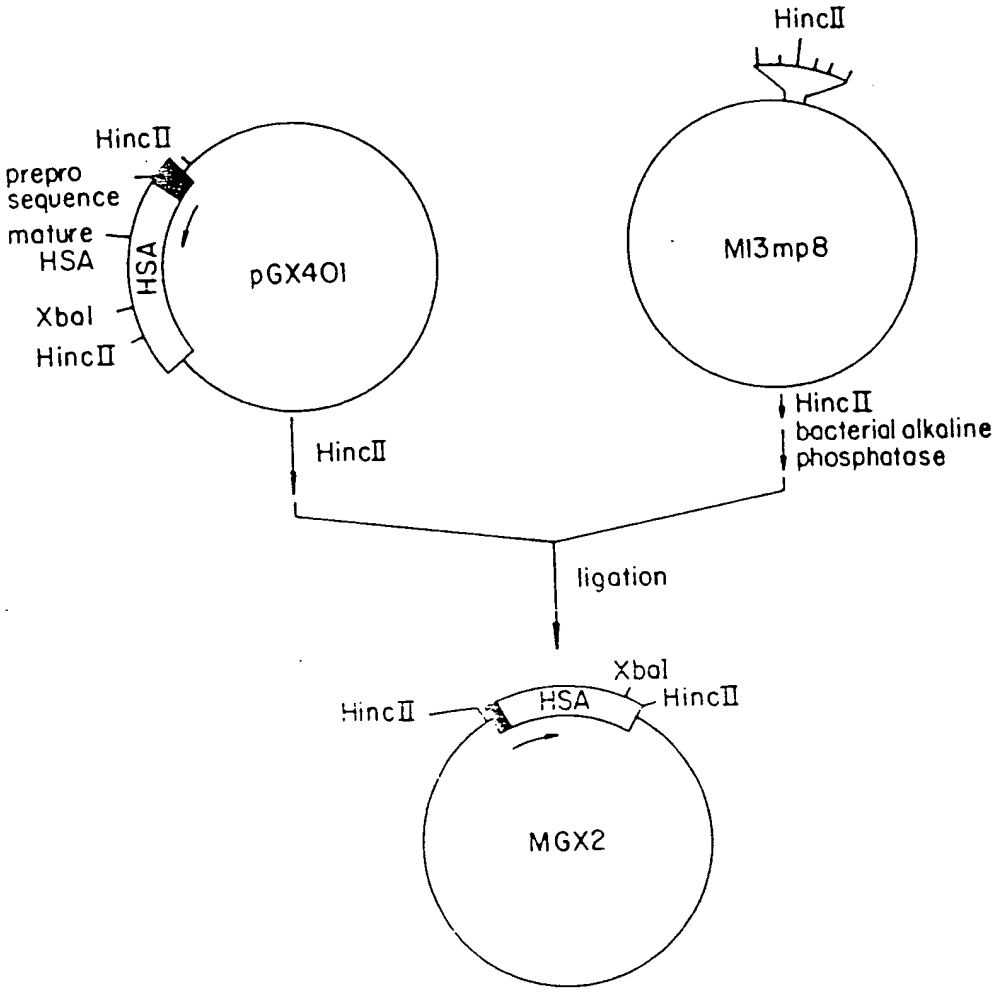

HincII
prepro sequence
mature HSA
HSA
XbaI
HincII
pGX401
HincII
HincII
M13mp8
HincII
bacterial alkaline phosphatase
ligation
XbaI
HincII
HSA
HincII
MGX2

FIG. 6

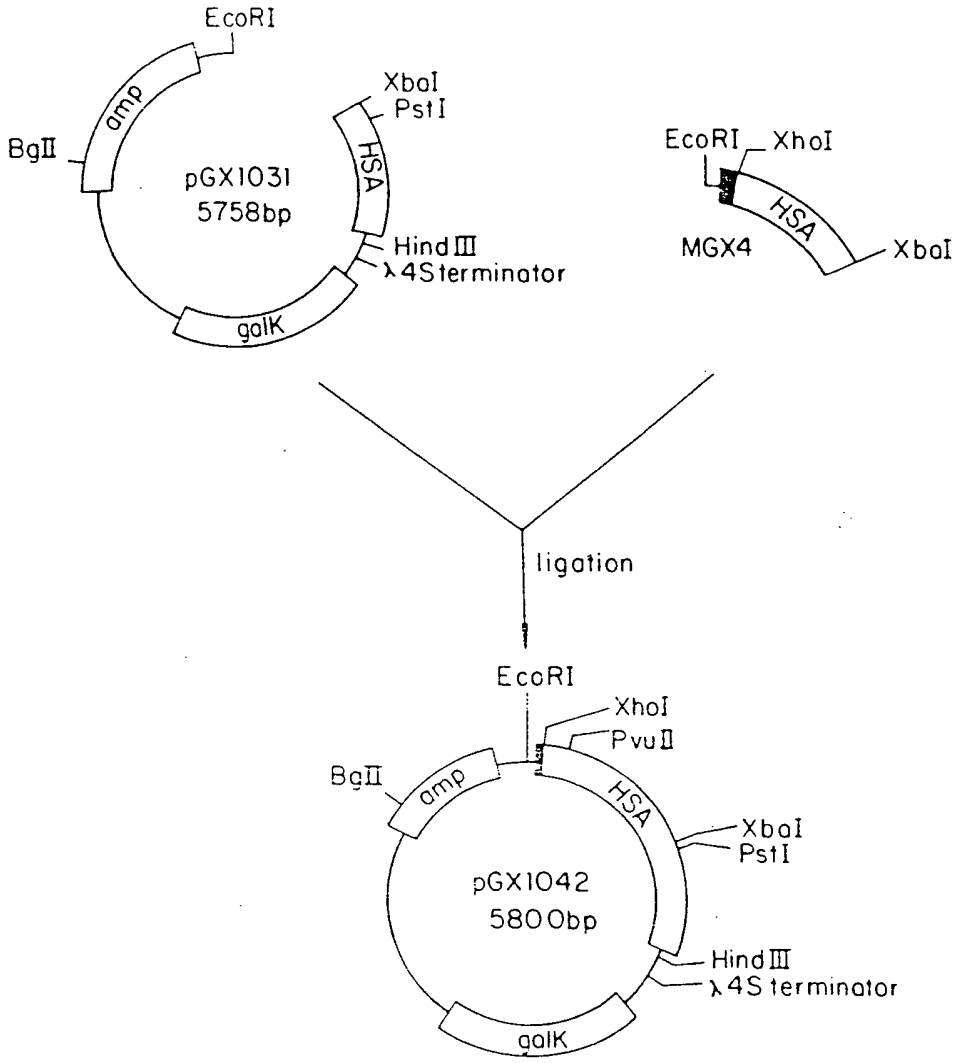
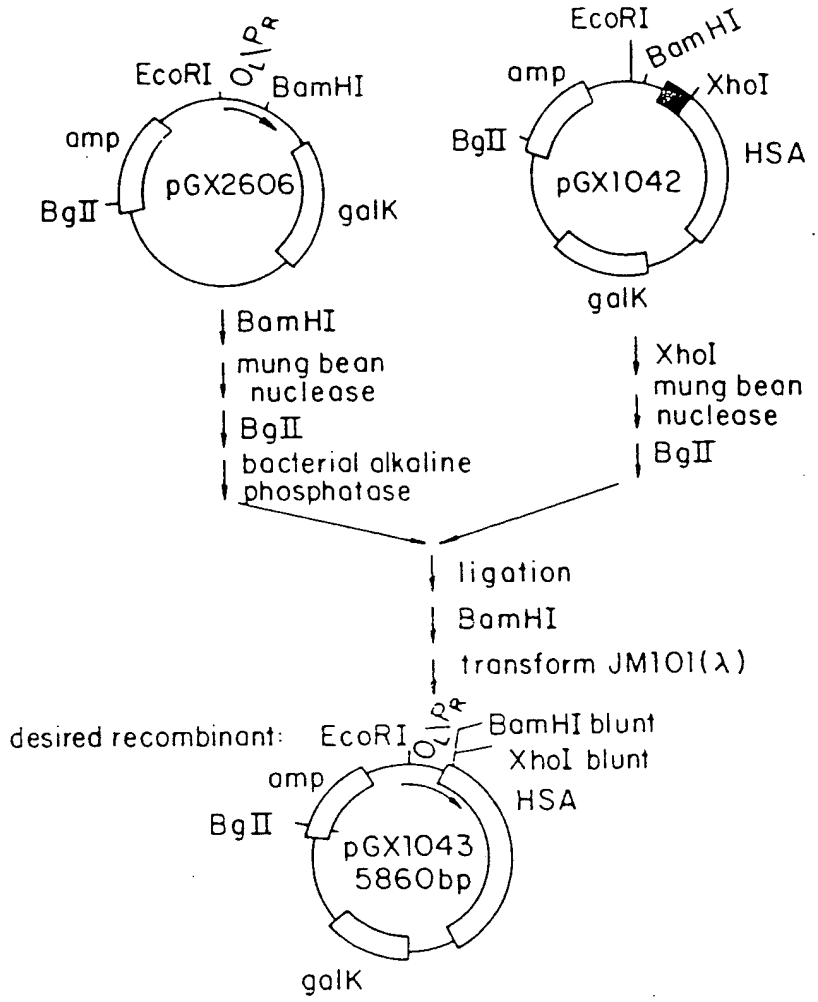

EcoRI
XbaI
PstI
BglII
amp
HSA
pGX1031
5758bp
HindIII
λ4Sterminator
galK
EcoRI
XhoI
HSA
MGX4
XbaI
ligation
EcoRI
XhoI
PvuII
BglII
amp
HSA
XbaI
PstI
pGX1042
5800bp
HindIII
λ4S terminator
galK

FIG.7



sequence of junction: TACTAAGGAGGTTGTA TG GAT GCA CAC

FIG. 8

European Patent Office

EUROPEAN SEARCH REPORT

0147198
Application number

EP 84 30 8981

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	CURRENT GENETICS, vol. 2, November 1980, pages 109-113; J.J. PANTHIER et al.: "Cloned beta-galactosidase gene of Escherichia coli is expressed in the yeast Saccharomyces cerevisiae" ---		
A	CHEMICAL ABSTRACTS, vol. 99, no. 19, 7th November 1983, page 164, abstract no. 153176f, Columbus, Ohio, US; B.A. CANTWELL et al.: "Molecular cloning and expression of a Bacillus subtilis beta-glucanase gene in Escherichia coli", & GENE 1983, 23(2), 211-19 ---		
P	CHEMICAL ABSTRACTS, vol. 101, no. 23, December 1984, page 153, abstract no. 205283v, Columbus, Ohio, US; E. HINCHLIFFE et al.: "Expression of the cloned endo-1,3-1,4-beta-glucanase gene of Bacillus subtilis in Saccharomyces cerevisiae", & CURR. GENET. 1984, 8(6), 471-5 ---		TECHNICAL FIELDS SEARCHED (Int. Cl.4)
	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY (USA), vol. 81, 1981, page 116, ref. H19; S.K. PICATAGGIO et al.: "The cloning of trichoderma reesei genomic DNA in Escherichia coli HB101" -----		

This present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	11-07-1986	DESCAMPS J.A.

CATEGORY OF CITED DOCUMENTS

- particularly relevant if taken alone
- particularly relevant if combined with another document of the same category
- technological background
- non-written disclosure
- intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

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(54) **Fermentation processes and their products.**

(57) The invention provides a process for the production of ethanol and a protein or peptide which is heterologous to yeast which comprises fermenting an aqueous sugar-containing medium with an industrial yeast strain which has been genetically modified to be capable of expressing a heterologous protein or peptide, recovering the ethanol so-formed, and obtaining the said heterologous protein or peptide from the fermentation products. The process may be applied to the industrial production of alcoholic beverages such as beer or distilled alcohol. The yeast inevitably obtained as a by-product in the process has improved value because of the heterologous protein or peptide which it contains and provides a source of the latter. Heterologous protein and peptides which may be produced by the new process include enzymes such as beta-lactamase, beta-glucanase and beta-galactosidase and proteins of therapeutic value such as human serum albumen.

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.)
P,X	BREWERS' GUARDIAN, September 1984, pages 34-37; R.S. TUBB: "Genetic development of yeast strains" * Page 35, column 1, lines 47-49; page 37, column 1; figure 4 *	1	C 12 N 15/[illegible] C 12 C 11/[illegible] C 12 P 7/[illegible] C 12 P 21/[illegible] C 12 N 9/[illegible] C 12 N 9/[illegible]
A	GB-A-2 094 341 (MITSUBISHI CHEMICAL INDUSTRIES LTD.) * Page 3, lines 31-37; claims 1,19 *	1	
A	CHEMICAL ABSTRACTS, vol. 98, no. 7, February 1983, page 179, abstract no. 47922r, Columbus, Ohio, US; R.A. IRVING et al.: "Development of an amylolytic Saccharomyces cerevisiae by genetic engineering", & PROC.-BIOENERGY R & D SEMIN 1982, 4th, 541-5		
A	EP-A-0 096 491 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA)		
A	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 47, no. 11, November 1983, pages 2689-2692; I. YAMASHITA et al.: "Molecular cloning of a glucoamylase-producing gene in the yeast Saccharomyces"		

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TECHNICAL FIELDS SEARCHED (Int. Cl.)

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FERMENTATION PROCESSES AND THEIR PRODUCTS

This invention relates to fermentation processes and their products, and more particularly to the production of alcohol, i.e. ethanol, by fermentation of sugars with yeast.

In the manufacture of alcohol by fermentation, sugars in aqueous solution are converted into ethanol by fermentation with yeast. The yeast grows during the fermentation and although a small proportion of the yeast may be used in a subsequent fermentation process, the remainder of the yeast constitutes an excess that must be disposed of. While this excess yeast has some uses e.g. in animal feedstuffs and the manufacture of yeast extracts, the quantity of excess yeast produced is large and its market value is relatively low.

Large scale fermentations of this kind fall into three broad categories:

(1) Fermentations in which the fermented aqueous medium obtained is the desired end product. Into this category fall ordinary brewing processes for the production of beer (a term which, as used herein, includes ales, stouts, lagers and other fermented drinks based on malt), cider and other fermented drinks.

(2) Fermentations in which the desired end product is a distilled drinkable alcoholic concentrate. Into this category fall fermentations for production of whiskies, brandies and other spirits, and alcohol for use in fortifying other drinks.

(3) Fermentations for the production of alcohol for industrial use. Into this category falls fermentations carried out in some countries on a large scale for the production of fuel alcohol.

The production of excess yeast is a characteristic of all these industrial processes.

Considerable interest has been shown in recent years in the genetic modification of microorganisms so that they become able to produce heterologous proteins and peptides, that is to say proteins and peptides which are not produced by their natural genetic constituents. A variety of microorganisms have been used for such genetic manipulation, and, amongst these, yeasts have attracted a certain amount of interest. However, yeasts used in laboratory experiments are not normally the same as the yeasts used in large scale industrial fermentations involving the production of alcohol, and the conditions of growth of yeast in the laboratory are very different from those encountered by yeasts in an industrial alcoholic fermentation.

The present invention is based on the discovery that it is possible to use, in an industrial fermentation involving the production of alcohol, genetically modified yeast capable of expressing a heterologous protein or peptide. Surprisingly, it has been found that the use of such yeast is compatible with industrial fermentation conditions. This means that the excess yeast obtained in the fermentation provides a source of the heterologous protein or peptide and thus has much enhanced industrial value. Further, since the alcohol product remains the principle objective of the fermentation, and the conventional equipment can largely be used with little alteration, the additional cost of producing the higher value yeast product is small, so that the new process may provide an economically viable route to heterologous proteins or peptides which, although valuable, do not command a premium price.

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The present invention accordingly provides a process for the production of ethanol and a protein or peptide which is heterologous to yeast which comprises fermenting an aqueous sugar-containing medium with a yeast strain which has been genetically modified to be capable of expressing a heterologous protein or peptide, recovering the ethanol so formed, and obtaining the said heterologous protein or peptide from the fermentation products.

The yeasts are a group of lower eukaryotic micro-organisms showing biological and biochemical diversity. In common usage the term "yeast" is used to describe strains of Saccharomyces cerevisiae that have commercial value in baking, brewing and distilling. Related yeasts are used in wine making and sake brewing, as well as in the production of fuel alcohol from sucrose or hydroysed starch.

All the yeasts used for brewing, baking and distilling may be taxonomically classified as Saccharomyces cerevisiae. Included within this classification are the top fermenting ale yeasts (S. cerevisiae) and the bottom-fermenting lager yeasts (S. uvarum or S. carlsbergensis).

In a strict sense brewers yeast is differentiated from all other yeasts in that it is a yeast strain which is used to make beer, i.e. a strain of yeast used currently in a beer manufacturing process. Such yeasts must be able to produce a palatable acceptable beer by their fermentative action upon hopped malt extract (brewers wort). The primary products of this fermentation are ethanol and carbon dioxide, which are essential constituents of beer. However, not all yeasts belonging to the species S. cerevisiae are capable of

fulfilling these requirements. Indeed, the critical factor in this respect is believed to be the ability of the yeast strain to form in subtly balanced proportions, quantitatively minor metabolic products such as esters, acids, higher alcohols and ketones. A yeast may be unsuitable for brewing because one or more of these minor metabolic products is produced in excessive amounts, either in absolute terms or relative to one another. (Rainbow, C.A., 1970, In "The Yeasts", eds, Rose, A.H. & Harrison J.S. Vol. 3, p. 147). .

In a general sense brewers yeast is differentiated from other yeasts by the properties which it possesses. Most strains of industrial yeast, unlike laboratory yeast, are incapable of undergoing mating; they are said to be homothallic. Industrial yeasts are usually aneuploid or polyploid, and there is therefore a reduced incidence at which gene mutations are phenotypically detected. Most polyploid strains do not sporulate or they produce spores of very low viability, thus frustrating any meaningful genetic analysis. These factors together tend to confer a measure of phenotypic stability on industrial yeasts which may contribute to their selection for industrial application. Similarly gene dosage which is associated with high ploidy may contribute to the general fitness of such strains for fermentation as compared to haploids and diploids, which generally ferment poorly.

In addition, brewers yeasts have certain technological behaviour which equips them well for interacting with their normal environment, brewers' hopped wort.

The manner in which the new process is operated depends on the type of industrial fermentation. Where the fermentation is designed to produce an

aqueous potable liquid such as beer, at the end of the fermentation, the fermented liquid is separated from the yeast (and normally any other solid material present in the fermented medium). In these circumstances, it is clearly desirable, and indeed normally essential, that the heterologous protein or peptide shall not become dissolved in the fermented liquid, since it is normally unacceptable for the heterologous protein or peptide to be present in a liquid which is to be drunk. In such circumstances, the heterologous protein or peptide may be obtained from the yeast cells. Where, however, the alcohol is recovered by distillation, as is the case in the second and third types of industrial fermentation mentioned above, it may be acceptable, and even desirable, for the protein or peptide to be present in the fermented liquid in dissolved form.

The yeast strain used in the new process must, of course, be suitable for the type of industrial fermentation contemplated. This objective may be secured by carrying out the genetic modification on a yeast strain which is known to have the desired characteristics, since it has been found that the desirable characteristics which make a yeast strain suitable for a particular type of industrial fermentation are not normally lost during the genetic modification. For example, where the fermentation is one for producing beer, the yeast strain chosen for genetic modification is preferably a known strain of brewers' yeast currently used in such fermentations. As already noted, such industrial strains of brewers yeast have characteristics different from those of "laboratory yeast", including in particular the ability to ferment hopped brewers wort.

Brewers wort is essentially a hot water extract of malted barley or other grains prepared by steeping and germination and flavoured with hops. The most important parameters with respect to yeast growth and metabolism are carbohydrate and nitrogen (and amino acid) composition. These vary from country to country and brewery to brewery, see, e.g., "Malting and Brewing Science", Vol. 2, Hopped Wort and Beer; by Hough, J.S., Briggs, D.E., Steven R. and Young, T.W., 1982, Chapman and Hall, London and New York, p.456-498. In general it may be said that brewers wort contains 5 to 10 g of total fermentable sugars per 100 ml of wort, at least half of which is maltose.

Additional factors which influence yeast growth and performance are: (1) Growth factors. These include substances like biotin, thiamine, riboflavin, pyridoxine, pantothenic acid and nicotinic acid. In general brewers wort is a rich source of these factors, which are depleted during yeast growth. (2) Minerals. The mineral rqeuirements of brewers yeast resemble those of most living organisms. Brewers wort meets these requirements, sup trace amounts of metal ions such as iron, potassium, magnesium, zinc, manganese and copper, which are essential for vital metabolic enzymes.

The most significant difference between a laboratory culture medium and a brewers wort is the sugar composition of the medium. Most laboratory media utilise glucose as the chief source of carbohydrate, whereas maltose is the chief fermentable constituent of wort.

Brewery fermentations normally take the form of anaerobic (oxygen free) fermentations. However, oxygen is a prime requirement for yeast growth in the initial stages of fermentation. Most laboratory fermentations are designed to maximise the yeast

0147198

biomass yield, whereas beer fermentations concentrate upon ethanol yield and product flavour. Thus the inoculation rate ("pitching rate") of a beer fermentation is higher than would normally be used in the laboratory. Consequently, the number of cell doublings (cell generations) is reduced to between 2 and 4 per fermentation.

The fermentation of beer wort is normally carried out at a temperature within the range of 8 to 25°C, a temperature at the upper end of this range, e.g. 15 to 25°C being used when the product is ale, and a temperature of e.g. 8 to 15°C being used where the product is lager. Under laboratory conditions, yeasts are cultivated at significantly higher temperature, e.g. 25 to 35°C.

Similarly, where the industrial fermentation is one for the production of alcohol which is separated by distillation, it is necessary to use genetically modified yeast obtained from a strain suitable for such fermentation. In such fermentations, the source of sugars may be, for example, grain, potatoes, c sugar cane, or sugar beet and may optionally have been pre-treated, e.g. by chemical or enzymic hydrolysis, to convert cellulose and/or starch therein into fermentable sugars.

The genetic modification of yeast may be effected in a known manner. Suitable methods are described in the literature, and particular methods are given in the Examples below.

A wide range of heterologous proteins or peptides may be chosen for expression in the yeast. By way of example mention may be made of enzymes such as beta-lactamase, beta-glucanase, and beta-galactosidase. Other useful heterologous

proteins and peptides include materials of human origin and/or useful in therapy, such as human serum albumin and immunoglobulins. Methods are described in the literature for the genetic modification of microorganisms to enable them to express such proteins and peptides.

The heterologous protein or peptide made available by the genetically modified yeast may be used in several different ways. In the simplest case, the protein or peptide is retained by the yeast in the yeast cells and the latter are used as such. Normally, however, it is preferred to isolate the heterologous protein or peptide. Where the latter is excreted by the yeast into the surrounding medium, the fermented medium is worked up for isolation of protein or peptide. As already noted, this method is normally unsuitable where the fermented medium is to be consumed, e.g. as a beverage. In such a case, the desired protein or peptide is obtained from the yeast produced during the fermentation. For example, the yeast cells may be ruptured to release their contents, and the protein or peptide then isolated from the latter.

The following examples illustrate the invention in more detail. The accompanying drawing shows gene maps illustrating the formation of a plasmid used in one example. These examples describe the modification of brewers yeast so that it produces the heterologous proteins beta-lactamase and/or beta-glucanase and the use of the modified yeast in a brewing process.

ß-lactamase is the name given to a group of proteins that constitute enzymes operative to catalyse the hydrolysis of the amide bond in the ß-lactam ring of 6-amino-penicillanic acid or 7-amino-cephalosporanic acid and of their N-acyl derivatives. Such derivatives are penicillins and cephalosporins, generally known as ß-lactam antibiotics (Citri, N., 1971, "The Enzymes", 3rd edition, ed. Boyer, P.A., IV, p 23).

ß-lactamase is widespread amongst the various bacterial species, being found in both Gram-negative and Gram-positive bacteria. The gene specifying the production of ß-lactamase has been variously assigned to both chromosomal and extrachromosomal elements. In enteric bacteria a ß-lactamase gene can frequently be acquired by infection with an extrachromosomal particle in the form of a plasmid and constituting a resistance factor (or R-factor). One such R-factor carrying a ß-lactamase gene, and thus conferring resistance upon its host bacterium to ß-lactam antibiotics, is R1 (Meynell, E. & Datta, N., 1966, Genetical Research, 7, p 134). This plasmid was identified in a clinical isolate of Salmonella paratyphi B (Meynell, E. & Datta, N., 1966, Genetical Research, 7, p 134). The species specificity of ß-lactamase has been brought into question since R-factors are capable of mediating their own transfer, and thus the transfer of the ß-lactamase gene among the Enterobacteriaceae (enteric bacteria) (Datta, N. & Richmond, M.H., 1966, Biochemical Journal, 98, p 204).

With the advent of genetic engineering (recombinant DNA technology) there has developed a requirement for easily manipulated plasmid vectors for use in DNA

cloning. The ß-lactamase gene present on plasmid R1 has been introduced into new plasmids in the construction of novel cloning vectors. One such vector is RSF 2124 (So, M. et al., 1975, Molecular and General Genetics, 142, p 239) constructed from the plasmid Col E1 and a derivative of R1, R1 drd 19 (Meynell, E. & Datta, N., 1967, Nature, 214, p 885).

RSF 2124 has been manipulated subsequently to produce the plasmid vector pBR322 (Bolivar, F. et al, 1977, Gene, 2, p 95), which has been further manipulated to form pAT153 (Twigg, A.A. & Sherratt, D., 1980, Nature, 283, p 216). All these plasmid vectors retain the ß-lactamase gene of R1 and are capable of specifying the production of ß-lactamase enzyme in Escherichia coli.

Additional manipulation of plasmid cloning vectors derived from pBR322, and therefore possessing the ß-lactamase gene of R1, has been necessary to construct plasmids capable of transforming yeast (i.e. of being introduced into yeast). Thus, for example, the plasmid pAT153 (Twigg, A.J. & Sherratt, D., 1980, Nature, 283, p 216) has been attached to segments of yeast chromosomal DNA (LEU-2 gene of Saccharomyces cerevisiae specifying the production of ß-iso-propyl-malate-dehydrogenase, an enzyme involved in the biosynthesis of leucine) and 2µm plasmid DNA (2µm is an endogenous plasmid of yeast) to form plasmid pJDB207 (Beggs, J.D., 1981, "Molecular Genetics in Yeast", eds. von Wettstein, D., Stenderup, A., Kielland-Brandt, M. & Friis, J., Alfred Benzon Symposium No. 16, Munksgaard, Copenhagen, p 383).

ß-lactamase was the first heterologous protein to be expressed in S. cerevisiae (Hollenberg, C.P., 1979, ICN-UCLA Symposium Molecular and Cellular Biology, 15, p 325; Hollenberg, C.P., 1979, "Plasmids of Medical, Environmental and Commercial Importance", eds. Timmis, K.N. & Puhler, A., Elsevier, p 481). The bacterial ampicillin-resistance gene specifying the production of ß-lactamase enzyme originated from plasmid pBR325, a derivative of pBR322, and therefore ultimately from Salmonella paratyphi B (see earlier references). The ß-lactamase protein synthesised in S. cerevisiae has been purified 100-fold over crude extracts, and its enzymic activity, molecular weight and binding to specific antibodies have been shown to be indistinguishable from the purified protein from E. coli (Roggenkamp, R. et al, 1981, PNAS USA, 78, p 4466). The level of ß-lactamase expression in yeast is low due to the weak function of the bacterial gene promoter (control region of the gene); however, gene expression can be greatly enhanced by the use of the ADH1 promoter (alcohol dehydrogenase) of yeast (Hollenberg, C.P. et al, 1983, "Gene Expression in Yeast", eds. Korhola, M. & Vaisanen, E., Proceedings of the Alko Yeast Symposium, Helsinki, p 73).

Yeast transformation (that is the introduction of DNA into yeast) can be a relatively inefficient process, with success depending upon a suitable selection system. Most plasmids currently in use for yeast transformation are selectable, because they carry a wild-type gene which complements an auxotrophic mutation in the chosen recipient strain which has been a laboratory haploid strain of S. cerevisiae. However, brewers' yeasts are prototrophic and have no auxotrophic requirements. To select transformants in brewers' yeast it is necessary to have a dominant gene conferring the ability to grow in otherwise adverse conditions. CUP-1 is a dominant yeast gene, specifying the production of a protein capable of chelating copper ions. This gene has been cloned on the yeast/E. coli shuttle vector pJDB207, by insertion of restriction-endonuclease-Sau3A-generated DNA fragments from strain X2180-1A to form plasmid pET13:1 (Henderson, R.C.A., 1983, "The Genetics and Applications of Copper Resistance in Yeast", Ph.D. thesis, University of Oxford). A genetic map of pET13:1 is included in the accompanying drawing. Plasmid pET13:1 carries the LEU-2 and CUP-1 chromosomal genes of yeast and the 2µm yeast plasmid origin of DNA replication as well as DNA derived from plasmid pAT153; consequently pET13:1 harbours the bacterial ß-lactamase gene which is known to express ß-lactamase in yeast. Henderson (1983) describes in some detail methods for transforming brewers' yeast (ale yeast and lager yeast) with plasmid pET13:1. He also described the screening of brewers' yeast transformants for ß-lactamase activity using a starch iodide plate assay described below. Clearly the bacterial ß-lactamase protein is produced in brewers' yeast transformed with pET13:1 and can be detected when transformants are grown upon the appropriate indicator medium.

The genetic modification of a particular strain of brewers' yeast, by the introduction into it of the plasmid pET13:1, will now be described. The yeast used was NCYC 240, which is an ale yeast which is available to the public from the National Collection of Yeast Cultures (Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, England).

Before strain NCYC 240 could be transformed with plasmid pET13:1 (CUP-1/ß-lactamase) its sensitivity to copper was assessed. To this end, samples of NCYC 240 were patched on YED glucose or YED glucose agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, solidified with 2% w/v agar) and grown for 2 days at 28°C. They were then replica plated to NEP agar medium ($MgSO_4.7H_2O$ 2g/l, $(NH_4)_2SO_4$ 2g/l, KH_2PO_4 3g/l, $CaCl_2.2H_2O$ 0.25g/l, yeast extract 2g/l, peptone 3g/l, glucose 40g/l solidified with 2% agar. Naiki, N. & Yamagata, S., 1976, Plant and Cell Physiology, 17, p 1281) containing increasing concentrations of copper sulphate ($CuSO_4$). The strain tested did not grow on NEP containing 0.1mM $CuSO_4$. It was therefore concluded that in excess of 0.1mM $CuSO_4$ in NEP would be sufficient to select for copper resistant transformants of brewers' yeast.

Plasmid DNA of pET13:1 was isolated from the bacterium Escherichia coli K-12 strain JA221 (recA1, leuB6, trp E5, hsdR-, hsdM+, lacY. Beggs, J.D., 1978, Nature, 275, p 104) by caesium chloride/ethidium bromide equilibrium gradient centrifugation of cleared cell lysates using the method of Clewell, D.B. & Helinski, D.R. (1967, Proceedings of the National Academy of Sience, USA, 62, p 1159) with the modifications of Zahn, G. et al. (1977, Molecular and General Genetics, 153, p 45).

Samples of NCYC 240 were prepared for transformation with pET13:1 by each of two methods: (A) the method of Beggs, J.D. (1978, Nature, 275, p 104), and (B) the method described by Henderson R.C.A. (1983, "The Genetics and Applications of Copper Resistance in Yeast", Ph.D. thesis, University of Oxford) with the exception that the protoplasting enzyme used was Zymolyase (40µg/ml) (Kirin Brewery Co. Ltd.). 100µl of yeast spheroplasts produced by methods A and B were mixed with 15µl of pET13:1 DNA (approximately 250µg DNA/ml) and treated with polyethylene glycol (1ml 40% PEG 4000 in 10mM $CaCl_2$, 10mM Tris/HCl pH 7-6). After the treatment with polyethylene glycol, cells were spun down and gently resuspended in 500µl NEP glucose medium containing 1.2M sorbitol. Following incubation for one hour at 28°C, cells were added to 10ml of molten NEP glucose 3% agar containing 0.3mM $CuSO_4$ and 1.2M sorbitol. This was then poured onto NEP glucose 2% agar medium containing 1.2M sorbitol and 0.3mM $CuSO_4$. Transformation plates were incubated for four to five days at 28°C after which time yeast colonies arising on the selective copper medium were picked off and patched upon NEP glucose agar containing 0.3mM $CuSO_4$. These patched colonies were designated putative pET13:1 transformants, and were checked as described below to confirm that they were genuine brewers' yeast transformants. The frequencies of transformation for each of the two methods A and B for NCYC 240 were <4 transformants/µg DNA and 20 transformants/µg DNA respectively.

It is usual when attempting to confirm that a strain of yeast or bacteria is a genuine transformant to check for the presence of one or more genetic characters specified by the incoming plasmid DNA. The putative transformants described above were therefore assessed for high-level copper resistance and ß-lactamase activity,

since each phenotype (copper resistance/ß-lactamase activity) is specified by genes carried on the plasmid pET13:1. The following methods were employed:

(a) High-level copper resistance. Putative pET13:1 transformants growing as patches on NEP glucose agar + 0.3mM $CuSO_4$ were sub-cultured by replica plating to the same medium and NEP glucose agar + 1mM $CuSO_4$. Those patched colonies which grew on the media containing both 0.3mM and 1mM $CuSO_4$ clearly possess high-level copper resistance. This character is presumed to be a feature of plasmid transformants carrying CUP-1, since copy number regulates copper resistance in yeast (Fogel, S. et al, 1983, Current Genetics, 7, p 347). It is not unreasonable to expect plasmid transformants to have a high-level of copper resistance due to the multiple copies of the plasmid genome. Those patched colonies which showed high-level copper resistance were then subjected to the ß-lactamase test.

(b) The ß-lactamase test for detecting ß-lactamase produced by yeast strains carrying chimaeric yeast/E. coli plasmids is routinely applied to yeast transformants. (Chevallier, M.R. & Aigle, M., 1979, FEBS Letters, 108, p 179). The method described by Chevallier and Aigle (1979) is strictly adhered to and involves the following procedure:

The basis of the test is that penicillinase (ß-lactamase) hydrolyses penicillin giving a reducing compound, penicilloic acid. The reducing action of penicilloic acid is rendered visible by the decoloration of a deep blue iodine-starch complex incorporated into a solid agar medium. Thus, if ß-lactamase-producing strains are placed on

the test medium a white halo appears around the ß-lactamase-producing strain.

Test medium: Yeast nitrogen base (Difco) 0.65% w/v, glucose 0.1% w/v, soluble starch 0.2% w/v, agar 2% w/v, buffered with 0.02M phosphate at pH 6-7.

Soft agar test medium: as above, but with 1% w/v agar.

Reagent: 3mg/ml I_2; 15mg/ml KI; 0.02M phosphate buffer pH 7; 3mg/ml ampicillin.

Plates containing the test medium are patched with an inoculum of putative brewers' yeast transformant. They are incubated at 30°C for 18 hours. A mixture of 4ml melted soft agar test medium plus 1.5ml reagent is prepared. The mixture is stirred and gently poured over the test medium. Plates, which are deep blue, are left for 1 hour at 30°C and thereafter placed at 4°C. After about 24 hours any strain producing ß-lactamase shows a well defined white (colourless) halo, whereas control strains without plasmid show a very slight and limited decolouration. ß-lactamase-producing transformants are therefore clearly distinguished from strains which do not possess the ß-lactamase gene.

(c) Inheritable instability. A characteristic feature of yeast strains transformed with 2µm based plasmids such as pET13:1 and pJDB207 (pJDB207 being the parental plasmid of pET13:1), is that the plasmid is inheritably unstable. The consequence of this instability is that a small proportion of yeast cells within a population segregate plasmid-free daughter cells at cell division. In the case of

plasmid pET13:1, plasmid-free cells can be detected on the basis of their sensitivity to copper (NEP glucose agar + 0.3mM $CuSO_4$). Thus, copper-resistant transformants (see (a) above) are streaked on YED glucose medium and grown for 3-4 days at 27°C. Colonies arising on YED medium are then replica plated to the same medium and NEP glucose agar + 0.3mM $CuSO_4$. Colonies which have segregated the copper-resistant plasmid pET13:1 do not grow on the copper-supplemented medium. A variation of this method for evaluating the segregational phenotype of brewers' yeast transformants can be employed, in which putative transformants are first inoculated into NEP glucose medium (liquid medium without agar) and grown overnight at 27°C. The following day cells can be plated out on NEP glucose agar at a suitable dilution to obtain single colonies following incubation for three days at 27°C. Yeast colonies can then be replicated to NEP glucose agar and the same medium supplemented with 0.3mM $CuSO_4$. Those brewers' yeast transformants which possess plasmid pET13:1 can be distinguished from spontaneous copper-resistant derivatives on the basis of their ability to segregate copper resistance.

Methods (a), (b) and (c) are sufficient in combination to confirm whether a putative copper-resistant transformant is genuine. It is also preferable to study the cellular morphology of all putative transformants by light microscopy. A careful comparison of transformant with the parental strain (i.e. untransformed brewers' yeast) will indicate whether the transformant is in fact a genetically modified yeast or a contaminant.

Other methods for verifying plasmid transformants could be used if desired.

The yeast transformant thus obtained identified as NCYC 240 (pET13:1) was deposited at the National Collection of Yeast Cultures, Colney Lane, Norwich, NR4 7AU, United Kingdom, on December 12th 1984 under No. NCYC1545.

A single colony of NCYC 240 (pET13:1), which was verified as a true plasmid transformant by the methods described above, was grown on NEP glucose agar with 1mM $CuSO_4$ and inoculated into 200ml of NEP glucose supplemented with 0.2mM $CuSO_4$ (the liquid medium). The culture was incubated in a shake flask at 28°C for two days after which the full 200ml was inoculated into 5 litres of the same liquid medium. Cultures were grown in stirred flasks at 20°C for four days. 5 litre cultures were then diluted, each into approximately 45 litres of lager wort. The worts were fermented for seven days and the yeast was harvested and repitched into an ale wort prepared as follows.

95% ale malt and 5% crystal malt were mashed with South Staffordshire water at 65.5°C for 90 minutes. Hops were added to 36 EBU and caramel was added to 30 EBU. The mixture was boiled for 90 minutes at 1 bar and subjected to a whirlpool stand of 30 minutes. The specific gravity of the wort at collection was 1055° at 15°C.

The yeast was pressed and pitched at 1.5lb/barrel and the maximum fermentation temperature was 16°C. The beer was racked when the specific gravity had fallen to 1012°. The beer was conditioned at -1°C for 3 days. The beer was filtered and diluted at 1038° gravity, 1008 PG, 24 EBU bitterness and 20 EBU colour. The ethanol content was 4%. The beer was found to be acceptable to drink.

A sample of the beer was dialysed and then concentrated by freeze-drying. The freeze-dried beer was

assayed for ß-lactamase activity and it was found that there was no detectable ß-lactamase activity.

A similar procedure was followed with NCYC 240 lacking plasmid pET13:1 (i.e. unmodified NCYC 240), with the exception that the initial yeast culture in NEP glucose did not include copper sulphate. The beers produced by fermentation using both NCYC 240 and NCYC 240 (pET13:1) were judged to be essentially similar by routine Triangular Taste Test and Flavour Profile analyses (for a review of these methods see R.J. Anderson, 1983, Brewers Guardian, November, p 25).

During the course of beer production with both forms of yeasts, samples of the yeast concerned were analysed in order to estimate cell number and cell viability. The results showed that there was little or no difference between the yeasts in these respects. In the case of the modified yeast, the proportion of cells containing the plasmid (pET13:1) was measured and it was found that relatively few cells lost the plasmid. Other factors were also monitored during fermentation with both the modified and the unmodified yeasts. These were: the drop in specific gravity of the wort with time, the increase in the number of cells with time and the size of the final crop of yeast. It was found that for each of those factors there was no significant difference between the use of the modified and the unmodified yeast.

Some of the modified yeast produced in the fermentation process was made available for use in a further, similar brewing process, while the excess yeast provided a source of ß-lactamase.

The ß-lactamase content was assessed by means of a biological assay and by means of an enzyme assay. In

such assays cells are harvested by centrifugation for 10 minutes and resuspended in 0.1M phosphate/citrate buffer pH 6.5 and disrupted using a Braun homogenizer. Glass beads and cell debris are removed by centrifugation (8000 x g for 10 minutes) and the supernatant is recentrifuged (1000 x g for 30 minutes). In assaying the resulting cell-free extracts by the biological assay, penicillin sensitive E. coli cells are plated on soft agar containing 25µg/ml ampicillin. 25µl of extract of NCYC 240 and NCYC 240 (pET13:1) are spotted on these plates which are subsequently incubated at 37°C. Spots of NCYC 240 (pET13:1) show strong growth of bacteria in the vicinity of the spot, spots of NCYC 240 do not. This indicates that NCYC 240 (pET13:1) cells obtained from a beer fermentation produce a substance capable of degrading penicillin and allowing the growth of sensitive E. coli cells, whereas cells of NCYC 240 (unmodified) do not possess this activity. The activity can be attributed to ß-lactamase protein. Additional evidence that this activity can be attributed to a ß-lactamase protein in NCYC 240 (pET13:1) can be obtained from the results of enzymic assays. In the first of these assays a qualitative paper disc detection system is employed, in which samples of yeast cell extracts are spotted on to Cefinase discs impregnated with the chromogenic cephalosporin, Nitrocefin, which turns from yellow to red in the presence of a ß-lactamase (BBL Microbiology Systems, Beckton Dickinson and Company, Oxford) (C.H. O'Callaghan et al, Antimicrobial Agents and Chemotherapy, 1972, 1, p 283). Cell-free extracts of NCYC 240 (pET13:1) from a beer fermentation turn the discs from yellow to red, whereas extracts of NCYC 240 (unmodified) show no colour change on the disc, thus demonstrating the presence of ß-lactamase protein in NCYC 240 (pET13:1) but not in NCYC 240. The ß-lactamase activity in yeast cell extracts is quantified by using the same chromogenic

cephalosporin, Nitrocefin, and the method described by C.H. O'Callaghan et al (1972, Antimicrobial Agents and Chemotherapy, 1, p 283). Enzyme reactions are performed at 37°C in a 1cm cell containing a total volume of 1ml Nitrocefin solution (51.6µg of Nitrocefin 87/312 per ml in 0.05 M phosphate buffer, pH7) to which 20µl of cell-free yeast extract is added. The change in optical density of the reaction mixture is determined at 386 nm and 482 nm using a Beckman DU 7 spectrophotometer. In this way crude cell-free extracts of NCYC 240 (pET13:1) from a beer fermentation are capable of destroying 4.87 n moles of Nitrocefin 87/312 /min/mg protein at 37°C and pH 7.0 (protein estimates are obtained from the absorption of ultra violet light at 230 and 260 nm according to V.F. Kalb and R.W. Bernlohr (1977, Analytical Biochemistry, 82, p 362). Crude cell extracts of NCYC 240 (unmodified) and boiled extracts of NCYC 240 (pET13:1) (20 mins at 100°C) do not possess any ß-lactamase activity.

Procedures similar to those described above in detail in relation to NCYC 240 have also been carried out with a proprietary strain of brewers' yeast, and the results obtained were very similar.

There now follows a description of the modification of NCYC 240 to enable it to produce a different protein material, namely a ß-glucanase. An endo-1,3-1,4-ß-D-glucanase (EC 3.2.1.73) is an enzyme which catalyses the hydrolysis of alternating sequences of ß-1,3 and ß-1,4 - linked -ß-D-glucan, as in barley ß-glucan and lichenan. The unique action of this enzyme precludes its ability to hydrolyse repeating sequences of ß-1,3 - linked glucan, as in laminarin, and ß-1,4 - linked glucan, as in carboxymethylcellulose (Barras, D.R., 1969, In "Cellulases and Their Applications",

156th meeting of the American Chemical Society, Sept. 11-12, 1968, Atlantic City, p 105).

The Gram-positive bacterium Bacillus subtilis produces an extra-cellular endo-1,3-1,4-ß-D-glucanase which behaves in a similar fashion to that described above (Moscatelli, E.A. et al, 1961, Journal of Biologial Chemistry, 236, p 2858; Rickes, E.L. et al, 1962, Archives of Biochemistry and Biophysics, 69, p 371).

A chromosomal B. subtilis ß-glucanase gene has been isolated by gene cloning from a strain of B. subtilis entitled NCIB 8565 (Hinchliffe, E., 1984, Journal of General Microbiology, 130, p 1285). The active gene was found to reside upon a 3.5 kilo-base pair restriction-endonuclease-Eco RI-fragment of DNA, which expressed a functional enzyme in E. coli. The cloned ß-glucanase gene was shown to encode an enzyme specific for the hydrolysis of barley ß-glucan, and was found to be predominantly extracytoplasmic in location in E. coli (Hinchliffe, 1984).

More recently the cloned ß-glucanase gene has been located by deletion analysis on a 1.4 kb restriction endonuclease PvuI-ClaI DNA fragment. A similar location has been assigned to a B. subtilis ß-glucanase gene isolated from strain NCIB 2117 (Cantwell, B.A. & McConnell, D.J., 1983, Gene, 23, p 211). A more precise molecular characterization by DNA sequence analysis of the NCIB 2117 has recently been reported (Murphy, N. et al, 1984, Nucleic Acids Research, 12, p 5355).

Yeasts, including S. cerevisiae, produce several different types of ß-glucanase; however, none is able to hydrolyse ß-1,3-1,4 - linked glucan (Abd-El-Al, A.T.H. & Phaff, H.J., 1968, Biochemical Journal, 109, p 347). It

must therefore follow that yeast does not produce an endo-1,3-1,4-ß-D-glucanase. The cloned ß-glucanase gene of B. subtilis has therefore been introduced into S. cerevisiae, and it has been demonstrated that the gene is capable of encoding a biologically active protein in S. cerevisiae and that the enzyme activity is characteristic of that found in B. subtilis and E. coli (Hinchliffe, E. & Box, W.G., 1984, Current Genetics, 8, p 471). The expression of the cloned ß-glucanase gene in S. cerevisiae is inefficient, relative to the amounts of biologically active enzyme produced in both B. subtilis and E. coli harbouring the cloned ß-glucanase gene. However, the enzymic activity in yeast can only be detected in crude cell extracts of yeast harbouring the cloned gene (Hinchliffe, E. & Box, W.G., 1984). This may mean that the enzyme produced by yeast is incapable of being secreted from the cell and is intra-cellular in nature; unlike the enzyme produced by bacteria, which is extra-cellular.

To introduce the ß-glucanase gene of B. subtilis into brewers' yeast NCYC 240, use was made of the shuttle vector pET13:1, that can replicate in both E. coli and S. cerevisiae, as mentioned above. The 3.5 kb Eco RI DNA fragment present in plasmid pEHB3 was subcloned by in vitro re-arrangement into the single Bam HI site of pET13:1, as illustrated in more detail in the accompanying drawing. In the gene maps in the drawing the radially hatched arcs represent DNA from B. subtilis that carries the ß-glucanase gene (ßG), the broad, unfilled arcs represent chromosomal DNA indicating the location of LEU-2 and [illegible] genes, (Henderson, R.C.A., 1983, "The Genetics and Applications of Copper Resistance in Yeast", Ph.D thesis, University of Oxford), and the narrow arcuate black lines represent 2µm plasmid DNA and the thick arcuate black lines represent E. coli vector DNA

sequences. Treatment with T4 DNA ligase following Eco RI digestion of pEHB3 (Hinchliffe, E., 1984, Journal of General Microbiology, 130, p 1285) was performed under dilute DNA concentrations, thus favouring circularization of the two products of Eco RI digestion. One of those products is a circle of the DNA from the broad black arc of pEHB3. On digestion of the products with the restriction endonuclease BglII that circle was broken at the BglII site to form a 3.5kb linear fragment. Meanwhile pET13:1 was digested and the resulting linear fragment was ligated with the linear fragment from pEHB3, using T4 DNA ligase. That digestion and ligation were carried out at higher DNA concentrations, which favour recombination of the rearranged B. subtilis DNA in the Bam HI site of pET13:1 (Henderson, R.C.A., 1983, "The Genetics and Applications of Copper Resistance in Yeast", Ph.D. thesis, University of Oxford). Ligation occurs because the endonucleases Bam HI and BglII generate mutually compatible cohesive ends which join to form Bam HI/BglII hybrid sites which are not recognized by either Bam HI or BglII. Transformants were selected in E. coli strain HB101 as being ampicillin-resistant, tetracycline-sensitive and ß-glucanase positive in E. coli, thus enabling them to be distinguished from pEHB3 in E. coli. The orientation of insertion of the re-arranged Eco RI fragment in pEHB10 was determined by restriction endonuclease digestion followed by agarose gel electrophoresis. The new plasmid has been designated pEHB10.

Plasmid DNA was isolated from HB101 harbouring the hybrid plasmid pEHB10; this DNA was transformed into the brewers' yeast NCYC 240 as described previously. Resistance to copper was selected, as also described above. Plasmid transformants of NCYC 240 were verified by a combination of high-level resistance determinations

and ß-lactamase assays, thus NCYC 240 (pEHB10) was derived.

Single colonies of NCYC 240 (pEHB10), NCYC 240 (pET13:1) and NCYC 240 were inoculated into 200ml of NEP glucose (supplemented with 0.2mM $CuSO_4$ where appropriate). Cultures were incubated while being shaken at 27°C for 2 days, after which time they were inoculated each into the 2 litres of the same medium. After 3 days' growth at 27°C cells were harvested by centrifugation and washed twice in 0.1M phosphate/citrate buffer at pH 6.4 prior to cell disruption in a Braun homogenizer. Supernatants were prepared as described previously with the exception that each was dialysed overnight against 2 x 2l of 0.1M phosphate/citrate: pH 6.4. Crude cell extracts of the three NCYC 240 yeast were then subjected to ß-glucanase assays as described by Hinchliffe & Box (1984). These assays demonstrated ß-glucanase activity associated with cell-free extracts of NCYC 240 (pEHB10) (1.17 n moles of reducing sugar liberated from barley ß-glucan/min/mg protein at 40°C and pH 6.2), but no activity in cell-free extracts of either NCYC 240 (pET13:1) or NCYC 240.

The yeast transformant thus obtained identified as NCYC 240 (pEHB10) has been deposited at the National Collection of Yeast Cultures, Colney Lane, Norwich NR47AU, United Kingdom on December 12th1984 under No. 1546.

A sample of the NCYC 240 (pEHB10) yeast was grown in the manner described above and used in a brewing process similar to that described above in relation to NCYC 240 (pET13:1). The process yielded beer that was acceptable to drink and that contained substantially no endo-1,3-1,4-ß-D-glucanase. Yeast from the brewing process was shown to contain the plasmid pEHB10, specifying the production of ß-glucanase. (1 n mole reducing sugar liberated from barley ß-glucan/min/mg protein at 40°C and pH 6.4), so that part of it could be re-cycled (that is used in a subsequent brewing operation) and part of it could be used as a source of

the enzyme. Furthermore, crude cell extracts of NCYC 240 (pEHB10) derived from the brewing process contain β-lactamase enzyme activity (2.33 n moles of Nitrocefin 87/312 destroyed/min/mg protein at 37°C and pH 7.0) as well as β-glucanase enzyme activity. This demonstrates the feasibility of producing more than one heterologous protein at the same time in a genetically modified brewing yeast, such as NCYC 240.

Endo-1,3-1,4-β-D-glucanase obtained from B. subtilis is currently marketed as an enzyme preparation for use in the brewing industry in alleviating problems associated with the presence of unwanted β-glucan. The process described above may therefore be used to produce this enzyme for the same purpose.

CLAIMS

1. Process for the production of ethanol and a protein or peptide which is heterologous to yeast which comprises fermenting an aqueous sugar-containing medium with a yeast strain which has been genetically modified to be capable of expressing a heterologous protein or peptide, recovering the ethanol so formed, and obtaining the said heterologous protein or peptide from the fermentation products.

2. Process according to claim 1 in which the ethanol is recovered in the form of an aqueous potable liquid which is substantially free from yeast and from the said heterologous protein or peptide and which contains substantially all the water and ethanol of the said fermented medium.

3. Process according to claim 1 in which the ethanol is recovered from the said fermented medium in the form of an ethanolic distillate.

4. Process according to claim 2 in which the aqueous sugar-containing medium contains maltose as the major sugar present.

5. Process according to claim 4 in which the aqueous sugar-containing medium is a barley malt-based beer wort.

6. Process according to claim 2, 4 or 5 in which the fermentation is effected at 8 to 25°C.

7. Process according to claim 3 in which the aqueous sugar-containing medium is a fermentation medium for the production of potable distilled ethanol or power ethanol.

8. Process according to claim 7 in which the said medium is based on grain, potatoes, cassava, sugar cane, or sugar beet, optionally pretreated to convert cellulose and/or starch therein into fermentable sugars.

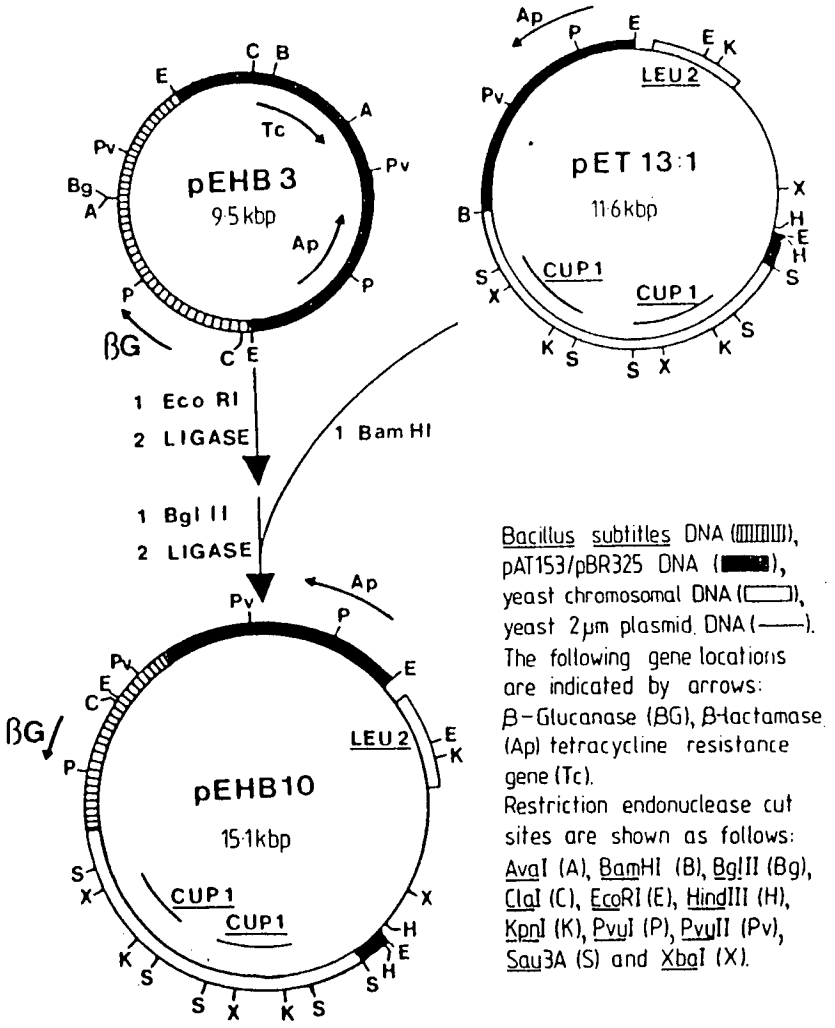
9. Process according to any one of claims 1 to 8 in which the fermentation is a substantially anaerobic fermentation.

10. Process according to any one of claims 1 to 9 in which the yeast used is a genetically engineered modification of an industrial strain of Saccharromyces cerevisiae, or S. carlsbergensis.

11. Process according to any of claims 1 to 10 in which the said heterologous protein or peptide is obtained as protein or peptide retained in the yeast produced during the fermentation.

0147198

CONSTRUCTION OF THE β-GLUCANASE CUP-1 PLASMID pEHB10



European Patent Office

Application number: 84308951.5
0147198

DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits:

NCYC 1545
NCYC 1546